

**Development of tools for surveillance of *Coxiella burnetii* in domestic ruminants and Australian marsupials and their waste**

Presented By

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13. SUPPLEMENTARY NOTES <b>The aim of this study was to develop improved methods to detect viable Coxiella burnetii in wastes from livestock production. A quantitative real-time PCR system (qPCR) with high sensitivity and specificity was developed to detect the C. burnetii in environmental samples associated with domestic ruminants and native Australian marsupials. Different detection chemistries and procedures were evaluated based on their sensitivity, specificity and reproducibility.</b>					
14. ABSTRACT <b>When combined, the IS1111a TaqMan qPCR and Geneworks PowerSoil DNA Extraction Kit provided a test which was capable of detecting as few as two C. burnetii genome equivalents in 0.2g of soil or faeces. Coxiella burnetii has been shown to display extreme resistance to environmental exposure. Therefore assessment of the viability of the organism in environmental matrices is more useful for risk assessment programs than detection of DNA alone. A quantitative reverse transcriptase PCR was developed that was able to detect viable C. burnetii cells in soil. The sensitivity of the assay was enhanced by heat-treating the soil samples prior to extraction of RNA. A system was developed to determine the efficacy of various disinfectant treatments against the environmental pathogen C. burnetii. Treatments evaluated included sodium hypochlorite, ozone, ultraviolet light peracetic acid (PAA), and Virkon S?. Sodium hypochlorite at a concentration of 0.1 mM reduced the infectivity of C. burnetii by over 92% while treatment with the same sodium hypochlorite concentration in wastewater showed significantly reduced efficacy. Despite this reduced potency, sodium hypochlorite is still useful for control of C. burnetii in the liquid waste of animal production. Commercially available ELISA and CFT assays exist for ruminants but there are no immunological tests available for detecting C. burnetii in marsupials even though Australian marsupials are known to be susceptible to C. burnetii. An indirect ELISA for detecting anti-Coxiella antibodies in kangaroos was developed. Paired serum and faecal samples were taken from 379 ruminants from Western Australia and the serum was tested with a commercially available ELISA and the complement fixation test while the faeces was tested using the qPCR developed during this study. Paired serum and faecal samples were taken from 343 kangaroos from WA and were tested with the antibody-ELISA developed during this study and by qPCR. A very low prevalence of anti-Coxiella antibodies was observed in the ruminants sampled and results from immunological tests correlated poorly with qPCR data. The development of an ELISA for use with kangaroo serum was problematic because of the lack of reference sera from 12/06/2009 School of Veterinary and Biomedical Sciences animals known to be infected with C. burnetii. Despite this results from the ELISA developed suggested that the apparent seroprevalence in the WA animals surveyed was approximately 34%. Results from testing kangaroo faeces with the qPCR correlated poorly with the results from the antibody-ELISA. These data suggest that kangaroos may be a significant reservoir of C. burnetii in Western Australia and due to cohabitation of kangaroos and domestic ruminants, may provide a link between the wildlife and domestic cycles of C. burnetii.</b>					
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## **Declaration**

The experiments in this thesis constitute work carried out by the candidate unless otherwise stated. The thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography and appendices, and complies with the stipulations set out for the degree of Doctor of Philosophy by Murdoch University.

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## Aims

1. To develop and apply quantitative polymerase chain reaction tools for the detection of *Coxiella burnetii* in waste from livestock production industries.
2. To develop a quantitative assay to assess the efficacy of disinfectants against *Coxiella burnetii* in liquid waste from livestock production.
3. To develop molecular tools to determine the viability of *Coxiella burnetii* recovered from wastes of livestock production.
4. To develop an enzyme-linked immunosorbent assay for the detection of anti-*Coxiella burnetii* antibodies in Australian marsupials.



## Thesis abstract

The aim of this study was to develop improved methods to detect viable *Coxiella burnetii* in wastes from livestock production. The impetus for this work arose because there is a significant risk of infection for humans attributed to contact with waste products from the livestock production industry. This situation is further compounded by the lack of suitable tools to detect viable *C. burnetii* in these wastes. In addition, effective disinfection strategies for livestock wastes are also required to reduce the risk of infection with *C. burnetii* for individuals that come into contact with these waste products.

A quantitative real-time PCR system (qPCR) with high sensitivity and specificity was developed to detect the *C. burnetii* in environmental samples associated with domestic ruminants and native Australian marsupials. Different detection chemistries and procedures were evaluated based on their sensitivity, specificity and reproducibility. Overall it was found that the TaqMan PCR targeting the *IS1111a* locus provided the most sensitive and reproducible test. The Geneworks PowerSoil™ DNA isolation kit provided the best compromise between reproducibility and recovery of DNA from livestock wastes. When combined, the *IS1111a* TaqMan qPCR and Geneworks PowerSoil DNA Extraction Kit provided a test which was capable of detecting as few as two *C. burnetii* genome equivalents in 0.2g of soil or faeces.

*Coxiella burnetii* has been shown to display extreme resistance to environmental exposure. Therefore, assessment of the viability of the organism in environmental matrices is more useful for risk assessment programs than detection of DNA alone. A quantitative reverse transcriptase PCR was developed

that was able to detect viable *C. burnetii* cells in soil. The sensitivity of the assay was enhanced by heat-treating the soil samples prior to extraction of RNA.

The factor most often associated with transfer of *C. burnetii* to humans is exposure to livestock or their waste. Therefore, decontamination of waste from livestock production industries is a key factor in preventing outbreaks of Q fever. A system was developed to determine the efficacy of various disinfectant treatments against the environmental pathogen *C. burnetii*. Treatments evaluated included sodium hypochlorite, ozone, ultraviolet light, peracetic acid (PAA), and Virkon S<sup>®</sup>. Sodium hypochlorite at a concentration of 0.1 mM reduced the infectivity of *C. burnetii* by over 92% while treatment with the same sodium hypochlorite concentration in wastewater showed significantly reduced efficacy. Despite this reduced potency, sodium hypochlorite is still useful for control of *C. burnetii* in the liquid waste of animal production.

Commercially available ELISA and CFT assays exist for ruminants but there are no immunological tests available for detecting *C. burnetii* in marsupials even though Australian marsupials are known to be susceptible to *C. burnetii*. An indirect ELISA for detecting anti-*Coxiella* antibodies in kangaroos was developed. Paired serum and faecal samples were taken from 379 ruminants from Western Australia and the serum was tested with a commercially available ELISA and the complement fixation test while the faeces was tested using the qPCR developed during this study. Paired serum and faecal samples were taken from 343 kangaroos from WA and were tested with the antibody-ELISA developed during this study and by qPCR. A very low prevalence of anti-*Coxiella* antibodies was observed in the ruminants sampled and results from immunological tests correlated poorly with qPCR data. The development of an ELISA for use with kangaroo serum was problematic because of the lack of reference sera from

animals known to be infected with *C. burnetii*. Despite this results from the ELISA developed suggested that the apparent seroprevalence in the WA animals surveyed was approximately 34%. Results from testing kangaroo faeces with the qPCR correlated poorly with the results from the antibody-ELISA. These data suggest that kangaroos may be a significant reservoir of *C. burnetii* in Western Australia and due to cohabitation of kangaroos and domestic ruminants, may provide a link between the wildlife and domestic cycles of *C. burnetii*.

## Abbreviations

<	less than
>	more than
≤	less than or equal to
≥	more than or equal to
±	plus or minus
%	percent
μ (prefix)	micro ( $10^{-6}$ )
p (prefix)	pico ( $10^{-9}$ )
°C	degrees Celsius
ABTS	2,2'-azino-di-(3-ethylbenzylthiazoline-6-sulfonate)
CFT	complement-fixation test
C <sub>T</sub>	cycle threshold
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine-tetra acetic acid, tri-potassium salt
ELISA	enzyme-linked immunosorbent assay
et al.	and others
FCS	foetal calf serum

<i>g</i>	unit of gravitational field
HP	highly pure
HRP	horseradish peroxidase
IFAT	indirect fluorescent antibody test
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
L	litre
LCV	large cell variant
LPS	lipopolysaccharide
m	metre
M	molar concentration
min	minute
mm	millimetre
ml	millilitre
NaOCl	sodium hypochlorite
NT	no treatment
NTC	no template control
OD	optical density

PAA	peracetic acid
PP	percent positive
qPCR	quantitative polymerase chain reaction
RT-qPCR	reverse transcriptase qPCR
SCV	small cell variant
SD	standard deviation
SDC	small dense cell
SEM	standard error of the mean
TE	Tris (hydroxymethyl) methylamine EDTA
TEN-T	TE and NaCl with 0.05% (v/v) Tween 20
UV	ultraviolet
WA	Western Australia
w/v	weight in volume
v/v	volume in volume



## 1. Literature review

### 1.1 Historical aspects

From 1933 onwards a series of acute febrile illness's were observed in workers from a slaughterhouse in Brisbane, Queensland, Australia who were serologically negative for infection with pathogens that were known, and could be tested for, at the time (Babudieri 1959). A consistent pattern to the infections was observed by Ted Derrick who was the chief investigator of the Brisbane outbreak. The similarities between individual cases indicated a common cause and in 1937 Derrick described a fever of unknown origin in meat workers in Queensland, Australia that was subsequently named 'Query' (Q) fever (Babudieri 1959; Williams and Sanchez 1994). The causative agent was initially thought to be a virus despite the fact that rickettsia-like organisms were identified in the spleens of infected laboratory animals (Reimer 1993).

Burnet and Freeman initially described the organisms isolated from clinical material from Q fever patients as a rickettsia, which led to the name *Rickettsia burneti* (Reimer 1993). At approximately the same time a group of researchers in Montana, USA, led by a rickettsiologist named Herald Cox, were investigating a tick-borne agent which resulted in a laboratory-acquired illness. They isolated a small Gram negative rod which they named *Rickettsia diaporica*. Subsequent investigation showed that the organism isolated by Cox was the same as the causative agent of the Brisbane outbreaks isolated by Burnet (Babudieri 1959). Continuing research by the Australian authors led to a greater understanding of the nature of Q fever including the organism's ability to infect wild animals which they thought might provide a natural reservoir for the disease (Derrick 1939; Smith



1940). Arthropod vectors were shown to be able to carry the Q fever agent (Smith 1940) and it was speculated that the tick *Haemophysalis humerosa* was involved in the sylvatic cycle of the organism (Derrick 1939) and could transmit infection to cattle (Babudieri 1959). Infection of humans was at that time thought to occur via tick bite or, perhaps, inhalation of contaminated dust. A laboratory epidemic of Q fever in 1940 demonstrated that the primary method of transmission was inhalation (Dyer, Toppings et al. 1940). The same study also implied that some instances of disease previously categorised as “primary atypical pneumonia” could in fact have been caused by Q fever.

Numerous epidemics of a febrile disease, given the name ‘Balkangrippe’, plagued German occupying forces in Europe during World War II (Babudieri 1959). After the end of the war these cases were retrospectively classified as Q fever (Babudieri 1959). The end of the war also appeared to coincide with the disappearance of the disease from the Balkans and Italy, which led to the hypothesis that the disease had been imported by American and Australian troops (Babudieri 1959). However, new outbreaks occurred in 1947 and 1948 in southern Germany and Italy that were later proven to be caused by *R. burneti* (Babudieri 1953).

In 1948 several phenotypic traits of the agent of Q fever led to the organism being removed from the genus *Rickettsia* and a new genus being established. To reflect the contributions made by both Burnet and Cox the genus was renamed *Coxiella* and the agent hence became *C. burnetii* (Reimer 1993). Since the late 1940’s interest in Q fever and advances in immunological and molecular techniques has stimulated a large amount of research into this disease and the aetiological agent (Babudieri 1959). More recently, the threat of bioterrorism

(Kagawa, Wehner et al. 2003; Welch, Baselski et al. 2003; Hassani, Patel et al. 2004; Beare, Samuel et al. 2006) has stimulated additional research in this area.

## 1.2 Bacteriology

### 1.2.1 Classification and related species

Since *Rickettsia burneti* was renamed *Coxiella burnetii* in 1948 (Philip 1948) it has remained the only definitively characterised member of that genus. Gene sequence analysis has led to placement of the *Coxiella* genus within the gamma subdivision of the proteobacteria in the order *Legionellae*, family Coxiellaceae but the genus is significantly removed from other members of the  $\gamma$ -proteobacteria (Seshadri, Paulsen et al. 2003). While *Coxiella* shares lifecycle and parasitic strategies with other members of the *Rickettsiae* and *Chlamydiae*, genome analysis has revealed that their genetic structures differ considerably in terms of metabolic capabilities, transporter profiles, the extent of genome reduction and the presence of mobile genetic elements (Seshadri, Paulsen et al. 2003). Instead, *Coxiella* is most closely related, in terms of phylogenetic analysis, to the pathogens *Rickettsiella* and *Legionella* (Weisburg, Dobson et al. 1989; Roux, Bergoin et al. 1997). Both *L. pneumophila* and *C. burnetii* multiply in host alveolar macrophage phagosomes and it has been demonstrated that they also contain similar *icm/dot* pathogenesis systems which assist intracellular multiplication (Zusman, Yerushalmi and Segal, 2003).

Recent investigations indicate that there are more members of the genus *Coxiella* but they are yet to be cultured or named. *Coxiella*-like organisms have been described from several species of ticks from varied geographical locations (Noda, Munderloh et al. 1997; Bernasconi, Casati et al. 2002; Mediannikov, Ivanov et al. 2003; Lee, Park et al. 2004; Reeves, Loftis et al. 2005). Reeves et al. (2005)

used genetic methods to characterise an agent found in bat ticks which showed a strong similarity to *C. burnetii*. However, results from standard PCR's performed to detect several *C. burnetii* genes were negative indicating that these genes are either lacking or have significantly different sequences in this novel bacterium. Similarly, a study in Korea identified a *Coxiella* spp., which possessed the *com1* gene but lacked the *icd*, *cbhE'* and *cbbE'* genes found in *C. burnetii*. These results indicate that *com1* is a genus specific gene whereas genes such as *icd*, *cbhE'* and *cbbE'* are specific for *C. burnetii* (Lee, Park et al. 2004). Bernasconi et al. (2002) found *Coxiella* species in eight ticks in Switzerland that were, with the exception of one of the isolates, identical to the endosymbiont identified by Noda et al. in 1997. This endosymbiont was distinguished from *Coxiella burnetii* using phylogenetic analysis of the 16S rDNA sequence (Noda, Munderloh et al. 1997). Homology of the 16S rDNA sequence of an organism isolated from *Haemaphysalis concinnae* ticks in far east Russia indicated that the organism was most closely related to *C. burnetii* and the organism isolated by Noda et al. (2007) (Mediannikov, Ivanov et al. 2003). A proposed novel species of *Coxiella* (*cheraxi*) was also isolated from the Australian redclaw crayfish (*Cherax quadricarinatus*) and showed 95.6% homology to *C. burnetii* based upon 16S rRNA sequences (Tan and Owens 2000). The authors proposed that this novel species of *Coxiella* may have diverged from *C. burnetii* approximately 200 million years ago. There has been some debate about the inter-strain homogeneity of *C. burnetii*. Using DNA-DNA hybridisation, Vodkin et al (1986) found little genetic variation between different isolates. Conversely, Thiele et al. (1993) found considerable heterogeneity in DNA restriction patterns of *C. burnetii* isolates using pulsed field gel electrophoresis.

### 1.2.2 Morphology

*Coxiella burnetii* is a pleomorphic obligate intracellular bacterium that replicates to high number, albeit slowly (Zamboni, Mortara et al. 2001), in the phagolysosomes of eukaryote phagocytic cells (Hackstadt and Williams 1981). Analysis of the complete genome of *Coxiella* Nine Mile phase I RSA493 showed 83 pseudogenes many of which were disrupted by only a single frameshift, which suggests that they are relatively recent developments. Thus it seems that *C. burnetii* is undergoing reductive evolution and that the obligate intracellular lifestyle of the bacterium may only be a recent innovation (Seshadri, Paulsen et al. 2003).

*Coxiella burnetii* has a cell wall structure that resembles other Gram negative bacteria in that it is tri-laminar with a dense intermediate layer which shares many common chemical elements with the peptidoglycan found in other Gram negative bacteria (Amano and Williams 1984). However, while *Coxiella* possesses enzymes required for the early stages of peptidoglycan synthesis it appears to lack enzymes that are needed to produce the final form of the polymer. This may indicate that *C. burnetii* obtains the necessary intermediates for formation of peptidoglycan from its host (Gonzales and Paretsky 1981). Furthermore, *C. burnetii* fails to take Gram stain well and gives a variable result when exposed to this stain (Gimenez 1965; McCaul and Williams 1981).

### 1.2.3 Phase variation

Antigenic variation similar to the rough/smooth phenomena seen in many Gram negative bacteria has been observed in various strains of *C. burnetii*. The most studied example is the Nine Mile strain which was isolated from ticks near Nine Mile River in Montana. Serial passage of this isolate through embryonated eggs resulted in a variant which was antigenically distinct from the organism that

was originally isolated (Stoker and Fiset 1956) and which seems to be at a selective advantage under *in vitro* conditions (Burton, Stueckemann et al. 1978; Moos and Hackstadt 1987).

The transition from “wild-type” phase I to non-pathogenic phase II in the Nine Mile strain is characterised by the gradual loss of the methylated sugars virenose and dihydrohydroxystreptose (Ftacek, Skultety et al. 2000; Slaba, Hussein et al. 2003) as well as many other sugar components of the “O” antigen side chains. Some components of the outer core of the LPS are also lost (Schramek and Mayer 1982; Schramek, Radziejewska-Lebrecht et al. 1985; Amano, Williams et al. 1987). The loss of various antigenic components of *C. burnetii* LPS appears to occur in a set, sequential order during serial passage of the organism through tissue culture with two “intermediate” forms between phase I and phase II (Hotta, Kawamura et al. 2002). While phase I LPS can be chemically reduced to the phase II form Ftacek et al (2000) observed that *in vitro* shortening of the O-polysaccharide chains does not occur, instead there is a redistribution of the existing LPS populations due to an increase in the number of cells expressing truncated O-chains. It seems that this redistribution of LPS populations results in the phase II form of *C. burnetii* binding much more strongly to host cell components than phase I (Williams, Peacock et al. 1981). Slaba and associates also found this with a series of experiments which showed that phase II LPS was more exposed than phase I LPS to components of the host's immune system (Slaba, Hussein et al. 2003). This could explain why phase II *Coxiella* is more easily cleared by the host's immune system. The lipopolysaccharide of *Coxiella* is a very important molecule in the defence of the organism against the host's immune system (Lukacova and Kazar 1996). Phase II variants of *C. burnetii* appear to have reduced virulence in comparison to phase I strains (Moos and Hackstadt 1987; Andoh, Russell-Lodrigue et al. 2005). Ormsbee *et al* (1978)

found that phase I was in fact 3000 times more infective than phase II in a guinea pig model. Conversely, it seems that phase II cells are significantly more infective for tissue culture cells than phase I cells (Moos and Hackstadt 1987; Baca, Klassen et al. 1993). Moos and Hackstadt (1987) also found that Nine Mile Crazy variant, which has intermediate length LPS, displays an intermediate level of infectivity. However, attachment and internalisation of *C. burnetii* appears to be a passive endocytotic event (Baca, Klassen et al. 1993).

In the Nine Mile strain the change from phase I to phase II LPS occurs with a concomitant chromosomal deletion. This deletion has been shown to span a region characterised by a high number of genes postulated to be associated with LPS and lipooligosaccharide (LOS) synthesis and general carbohydrate metabolism (Vodkin and Williams 1986; Hoover, Culp et al. 2002; Denison, Massung et al. 2007). It appears that for *C. burnetii* phase variation may provide a means to conserve energy in immunocompromised hosts by removing metabolically expensive structures on the cell surface that are required for host immune system evasion (Ftacek, Skultety and Toman, 2000). However, phase variation is not completely consistent between isolates in regard to the size and location of the chromosomal deletion and LPS-synthesis genes are not always involved (Vodkin and Williams 1986). Thompson et al. (2003) used sequencing and PCR with a comprehensive set of primers to search for chromosomal deletions in three strains, the M-44 Grita strain and the Australian strains QD and C5, which have been shown to have truncated LPS. The primers were designed to cover the region corresponding to the characteristic deleted region from the phase II Nine Mile strain. The authors' results indicated that none of the three strains examined contained deletions that would affect function in the same regions as the Nine Mile strain. Phase II isolates that have been shown to have

the deletion include NM phase II clone 4, NM phase II clone 1, NM Baca and RSA 514 (Denison, Massung et al. 2007).

#### 1.2.4 Lifecycle stages

McCaul and Williams (1981) proposed the names small cell variant (SCV) and large cell variant (LCV) for two morphologically distinct *C. burnetii* cells seen in persistently infected host cells. A third lifecycle variant called the small dense cell (SDC) has also been proposed (McCaul and Williams 1981), which is distinguishable from the SCV by the increased physical resistance it displays (McCaul, Banerjee-Bhatnagar et al. 1991). Both LCV and SCV have a typical Gram negative cell wall containing two distinct layers separated by the periplasmic space. The SCV differs in that the space between layers in its cell wall is filled with a material comprised of peptidoglycan and proteins and may explain the increased environmental stability of the SCV in comparison to the LCV (McCaul and Williams 1981). A later study demonstrated that the LCV had only 2% peptidoglycan by weight whereas the SCV contained 32% (Amano, Williams et al. 1984). It was proposed that incomplete cross-linking of peptidoglycan was responsible for the increased susceptibility of the LCV to lysis by osmotic shock and other environmental stressors (Amano, Williams et al. 1984). Furthermore, where the LCV is typically up to 2 µm in length, has dispersed chromatin and possesses a more rounded shape, the SCV is rod shaped with a length of about 0.45 µm and an electron-dense nucleoid (Nermut, Schramek et al. 1968). Both of these variants are metabolically active but the processing of glucose and glutamate is considerably lower in the SCV than the LCV (McCaul, Hackstadt et al. 1981). The existence of an *in vivo* *C. burnetii* endogenous spore was confirmed in 1994 by electron microscopic examination of three cardiac valves from different patients with Q fever endocarditis. Previously this life cycle variant had only been observed

*in vitro* (McCaul, Dare et al. 1994). Both SCV and LCV are capable of dividing by binary fission within an appropriate host cell.

Coleman et al. (2004) performed a temporal analysis of the differentiation of *C. burnetii* and found that *Coxiella*'s lifecycle followed a typical bacterial growth cycle. A lag phase followed infection of host cells during which time SCV's differentiated into LCV's. The LCV predominates during exponential growth which supports earlier work showing that this form is metabolically and replicatively active (Zamboni, Mortara et al. 2001). During the transition from exponential growth to stationary phase the population proportions changed such that SCV become more prevalent. This progression was paralleled by a decrease in metabolic activity (Coleman, Fischer et al. 2004) as was proposed by Zamboni et al (2001). During differentiation a sporulation-like process occurs where the septum is positioned asymmetrically to form a differentiated daughter cell (SCV) which is released from the mother cell following cell lysis (McCaul, Williams et al. 1991).

### **1.2.5 Tissue tropism**

*Coxiella burnetii* has been shown to have a strong affinity for mononuclear phagocytes and therefore can be found readily in the spleen, liver and bone marrow during the acute stage of Q fever (Baca and Paretsky 1983; Baumgartner and Bachmann 1992). Further evidence for this predilection was provided by a vaccination trial in mice, which showed that the spleens of the animals contained as many as 20-fold more *Coxiella* than the next most heavily infected tissue sampled (liver) (Zhang, Wen et al. 2005). In addition, the number of bacteria in spleen tissue remained elevated for 13 days once infection had been established but blood and lung tissue samples had relatively low levels of infection (Zhang,



Wen et al. 2005). This indicates that the spleen is the major site of *C. burnetii* replication in non-pregnant mice.

In pregnant animals it appears that *C. burnetii* displays a tropism for reproductive tissues. The trophoblast cells of chorioallantoic membrane were the first cells infected in the placenta of pregnant goats experimentally infected with *Coxiella* (Sanchez, Souriau et al. 2006). The same study also showed that massive bacterial multiplication occurred in the portion of the placenta that was associated with the foetus in the last week of pregnancy while the maternal portion remained relatively lesion free and contained only low levels of *Coxiella*. This great proliferation of bacterial cells in the foetal placentas coincided with abortion of the fetuses (Sanchez, Souriau et al. 2006). Another experimental infection of pregnant goats found that almost all aborted kids were PCR positive for *Coxiella* (Arricau Bouvery, Souriau et al. 2003). It has been hypothesised that there might be a correlation between hormonal levels of pregnant animals and increased *Coxiella* infection (Biberstein, Behymer et al. 1974), perhaps helping to explain the altered tissue tropism that occurs in pregnant animals. This argument is further strengthened by the presence of coding regions in the *C. burnetii* genome that are similar to eukaryotic genes for fatty acid desaturases and sterol reductases. This may indicate that *Coxiella* possesses a tissue or organ tropism for placenta or reproductive tissue (Seshadri, Paulsen et al. 2003).

#### **1.2.6 Resistance to chemical and physical stress**

The small cell variant of *C. burnetii* has been shown to be incredibly resistant to physical and chemical insult including elevated temperature and pressure, desiccation, osmotic shock and several chemical disinfectants (Ransom and Huebner 1951; Malloch and Stoker 1952; McCaul, Hackstadt et al. 1981; McCaul,

Banerjee-Bhatnagar et al. 1991; Cerf and Condron 2006). It has also been demonstrated that *C. burnetii* remains viable for 30 days in dried sputum; 120 days in dust; 49 days in dried guinea pig urine; 586 days in tick faeces; 42 months in milk stored at 4–6°C and 12-16 months in wool stored at 4-6°C (PHAC 2001). The resistance of the metabolically active LCV is less dramatic and the two variants can be distinguished by their respective resistance to osmotic shock, with the LCV undergoing plasmolysis (McCaul, Hackstadt et al. 1981; Williams, Peacock et al. 1981). The SCV and SDC also share some characteristics with the spore-forming bacteria *Bacillus subtilis*. These may contribute to the extreme resistance that it shows and include a low level of water in the spore core (Cortezzo, Koziol-Dube et al. 2004) and protection of DNA by saturation with DNA-binding proteins (Nermut, Schramek et al. 1968). In addition, the *C. burnetii* genome contains a gene with strong homology to the stationary phase regulator sigma factor of *Bacillus subtilis* (Seshadri and Samuel 2001).

The association of peptidoglycan and protease-resistant covalently bound proteins may in part explain the resistance to environmental stressors that *C. burnetii* exhibits (Amano and Williams 1984). However, the *Coxiella* genome contains no pathways for synthesis of storage compounds such as glycogen and polyhydroxybutyrate, which raises questions about how this pathogen remains viable in the extracellular environment for so long (Seshadri, Paulsen et al. 2003).

### **1.3 Pathogenesis and pathology of Q fever**

#### **1.3.1 Host immune response**

It has been demonstrated that cellular and humoral immune responses are involved in host resistance to infection by *C. burnetii*, both of which can be stimulated by challenge with viable cells or vaccination (Waag, England et al.

1997; Zhang and Samuel 2003). However, an experimental infection in immunodeficient mice showed that acquired immunity is essential for the host to clear infection by phase II *C. burnetii* because the innate immune response of the SCID mice was only sufficient to stop the replication of *Coxiella* but not sufficient to clear the infection (Andoh, Russell-Lodrigue et al. 2005).

Phagocytosis of infectious micro-organisms by polymorphonuclear leukocytes, monocytes and macrophages is accompanied by a respiratory burst resulting in production of reactive oxygen species (Salin and McCord 1975) which are thought to have microbicidal properties (McRipley and Sbarra 1967). It has been shown that this respiratory burst does not occur when neutrophils phagocytose *C. burnetii* cells and it appears likely that an absence of superoxide anions allows the pathogen to establish a persistent infection (Akpориaye, Stefanovich et al. 1990). Brennan et al. (2004) found that two major factors involved in the host's control of *C. burnetii* infection are reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). IFN- $\gamma$  stimulation results in activation of NADPH oxidase, a ROI, which reduces oxygen to a superoxide anion which can then enter the phagosome and subsequently be converted to hydrogen peroxide by a dismutase. The hydrogen peroxide can then break down the membrane of the *Coxiella* cells by disrupting protein channels in the outer membrane (Clark 1990). Both factors were found to inhibit replication in murine cell lines and mice (Brennan, Russell et al. 2004). Production of ROI and RNI is linked to the host's T cells activating enhanced biocidal mechanisms in macrophages (Asher *et al*, 1980, Izzo and Marmion, 1993). The cytokine response initiated by invading *C. burnetii* cells appears to be dependent upon the strain of *Coxiella* present. The Nine Mile strain induces less TNF $\alpha$ , IL-6 and IL-8 production compared to the Priscilla strain, which in turn induces less IL-1 $\beta$  and IFN- $\gamma$  than the Nine Mile strain. It seems that *C. burnetii*, partially through the LPS content of the cells, is able to selectively

stimulate or suppress groups of pro-inflammatory cytokines (Kuznetsova, Tokarevich et al. 2003). A factor specific to phase I *C. burnetii* cells but independent from LPS has also been found to have immunosuppressive characteristics (Waag and Williams 1988). Conversely, the chloroform-methanol residue (CMR) of *Coxiella* appears to stimulate non-specific humoral and cellular antiviral immunity, possibly via activation of antigen processing cells (Zvilich, Williams et al. 1995). It has also been shown that factors of *C. burnetii* promote a potent protective antibody response to lethal viral infections (Waag, Kende et al. 1990), protozoal agents (Clark 1979) and bacteria (Mika, Goodlow et al. 1954; Kelly 1977; Damrow, Cantrell et al. 1981). While the cellular component responsible for this phenomena has not been fully characterised it is believed to be associated with the lipopolysaccharide (LPS) of the pathogenic phase I form of the organism (Waag and Williams 1988). The non-specific immunity induced by *C. burnetii* is believed to be caused by sensitisation of the immune system, in particular the interferon response, leading to an enhanced response when challenged by an invading micro organism (Clark 1979; Hoebe, Janssen et al. 2003).

Helbig et al. (2005) suggested that the hosts' idiosyncratic immune response to *C. burnetii* determines progression to the acute form of Q fever. Helbig et al. (2005) performed a study to quantify the genetic relationship between HLA allele frequency and progression to chronic Q fever. They showed that Q fever fatigue syndrome (QFS) patients were more likely to carry HLA-DRB1\*11 compared to Q fever-negative controls, patients who made a complete and uncomplicated recovery from acute Q fever or sufferers of Q fever endocarditis (QFE) (Helbig et al. 2005). Only weak associations were observed between the QFE group and immune response genes (Helbig, Harris et al. 2005). Possession of HLA-DRB1\*11 and HLA-DRB1\*12 (DR5) molecules has been associated with lower

interferon gamma production and increased humoral T helper 2 responses (Petrovsky and Harrison 1997) and, as mentioned previously, production of interferon gamma is important for clearing *Coxiella* infections (Brennan, Russell et al. 2004).

In humans, antibodies to phase II *C. burnetii* antigen remain elevated for at least one year whereas anti-phase I antibodies are barely detectable using the complement fixation test (Enright, Longhurst et al. 1971). In addition, phase II antigen generally fixes complement more strongly compared to phase I antigen (Enright, Longhurst et al. 1971). These results were supported by similar data obtained with the immunofluorescence test. The study focused on 191 human patients who had presented with symptomatic Q fever following an outbreak of the disease in Switzerland (Dupuis, Peter et al. 1985). Conversely, a study on 30 Q fever-positive abattoir workers found that IgM to phase I antigen persisted for 27 weeks in one patient while IgM to phase II antigen was not detected beyond 17 weeks using an IgM immunofluorescence technique (Hunt, Field et al. 1983). In addition, phase II-specific IgM was only detectable for 10 weeks (Hunt, Field et al. 1983). In general Hunt et al. (1983) observed that phase I-specific IgM occurs with higher titres and persists for longer periods than phase II-specific IgM. Host levels of anti-phase II IgG have been shown to be elevated earlier than anti-phase I antibodies in infections in humans, guinea pigs and mice (Hunt, Field et al. 1983; Peacock, Philip et al. 1983; Peter, Dupuis et al. 1985; Moos and Hackstadt 1987; Li, Niu et al. 2005).

### **1.3.2 Pathogenesis**

Infection in animals and humans most often occurs via inhalation (De Lay, Lennette et al. 1950; Abinanti, Welsh et al. 1955; Tigertt, Benenson et al. 1961;

Riemann, Brant et al. 1975; Yanase, Muramatsu et al. 1998; Gardon, Heraud et al. 2001) and, after primary multiplication in the regional lymph nodes, a transient bacteraemia persists for five to seven days (Woldehiwet 2004). Subsequent progression of the infection may take one of several courses although the exact factors that contribute to disease differentiation remain speculative. The pathogenesis of *C. burnetii* has been associated with genetic characteristics identified by RFLP patterns, plasmid groups and strain type (Samuel, Frazier et al. 1985; Hendrix, Samuel et al. 1991; Russell-Lodrigue, Poels et al. 2005). Acute Q fever has been linked to genomic groups I, II and III whereas groups IV and V have been associated with the chronic form of the disease (Hendrix, Samuel et al. 1991). However, other studies refute that genetic characteristics of *Coxiella* determine the form of Q fever caused (Yu and Raoult 1994; Toman, Hussein et al. 2003) and suggest that disease outcome is more closely associated with host immune factors (Koster, Goodwin et al. 1985; Honstetter, Imbert et al. 2003). The interaction between host factors and disease outcome was highlighted in a study conducted by Raoult et al. (Raoult, Tissot-Dupont et al. 2000), which showed that progression from acute to chronic Q fever was strongly associated with aspects of physiology including pregnancy, vascular or valvular lesions, cirrhosis and cancer. However, the immunogenic characteristics of the invading coxiellae may also play a role in the development of the chronic form of Q fever. For example, considerable amounts of two branched sugars of the LPS (virenose and dihydrohydroxystreptose) of *C. burnetii* react strongly with phase I antibody and thus may be preferentially displayed during chronic infection (Slaba, Hussein et al. 2003).

Chronic *C. burnetii* infections may persist for months and perhaps even years (Marmion 1997) in both humans and animals (Baca, Scott et al. 1985; Marmion, Storm et al. 2005) and prior to onset patients may have undetectable levels of

antibody (Raoult, Tissot-Dupont et al. 2000). It has been suggested that long term persistence of *C. burnetii* in bone marrow may be universal following primary acute Q fever (Helbig, Harris et al. 2005). Given the predilection of *C. burnetii* for tissues that contain high numbers of mononuclear phagocytes (Baca and Paretsky 1983) it is reasonable to suggest that bone marrow would be one of the first sites to be colonised in a host. The viability and numbers of the pathogen may be regulated by the hosts' immune system except in a small percentage of infections, which may subsequently develop into Q fever fatigue syndrome (QFS), Q fever endocarditis (QFE), a reoccurrence of the original disease or osteoarthritis (Raoult, Bollini et al. 1989; Helbig, Harris et al. 2005). Of these Q fever endocarditis has the highest rate of mortality but QFS can also have a huge impact on the sufferer and their family due to the length of illness (possibly more than 10 years) and the lack of effective treatment and diagnostic tools (Marmion 1997). Chronic Q fever may take up to 20 years to develop and if misdiagnosed can do significant damage to heart valves and may lead to more significant health problems (Kimbrough, Ormsbee et al. 1981).

*Coxiella. burnetii* can be maintained *in vitro* for months or even years without addition of fresh uninfected tissue culture cells with little change in the growth characteristics of the host cells (Khavkin and Amosenkova 1981; Baca, Scott et al. 1985; Roman, Coriz et al. 1986). When an infected cell divides only one daughter cell receives the parasitised vacuole leaving the other daughter cell uninfected and susceptible to infection by *C. burnetii* (Roman, Coriz et al. 1986; Hechemy, McKee et al. 1993). The transfer of *C. burnetii* to only one daughter per mitotic division may play some part in chronic long-term Q fever infections.

In gram negative bacteria LPS is the most important virulence factor and the same appears to be true of *C. burnetii* (Baca and Paretsky 1983; Schramek,

Radziejewska-Lebrecht et al. 1985; Amano, Williams et al. 1987; Moos and Hackstadt 1987). The full length LPS of phase I *C. burnetii* isolates may provide protection from the hosts' immune system and prevent clearance during the bacteraemic stage of infection (Lukacova and Kazar 1996; Slaba, Hussein et al. 2003). *Coxiella burnetii* cells then gain access to human macrophages and monocytes by binding to the complex of leukocyte response integrin and integrin-associated protein on the cell surface and subsequently being internalised (Mege, Maurin et al. 1997; Seshadri, Paulsen et al. 2003). Internalisation is a passive phagocytic process although, for phase I cells, active subversion of the phagosome then occurs where phagolysosomal fusion is delayed, perhaps providing enough time for the pathogen to revert from the dormant small cell variant to the metabolically active large cell variant (Howe and Mallavia 2000). Following this delay the phagosome fuses with a lysosome to form a phagolysosome (Howe and Mallavia 2000). Coxiellae are acidophiles and thus the harsh acidic (pH 4.7-5.2) environment of the phagolysosome actually enhances *C. burnetii*'s metabolism (Hackstadt and Williams 1981; Hackstadt 1983; Zuerner and Thompson 1983; Baca, Roman et al. 1993). Survival of *Coxiella* in this harsh environment is thought to be aided by genes associated with stress-response and vacuole detoxification (Seshadri, Paulsen et al. 2003) and a high proportion of basic proteins that serve as a proton "sink" to buffer against the acidic environment in the phagolysosome (Seshadri and Samuel 2001). However, it seems that *C. burnetii* does not have an intrinsic resistance to other bactericidal conditions found in the host cells phagolysosome. Instead, coxiellae expend considerable metabolic effort to inhibit the respiratory burst (Clark 1990) through production of acid phosphatase (Baca, Roman et al. 1993). Fusion of the infected parasitophorous vacuole (PV) with other vacuoles may provide *C. burnetii* with fresh nutrients for growth and division (Howe, Melnicakova et al. 2003). Over a



period of approximately six days the population dynamics of the invading *C. burnetii* cells changes such that the metabolically dormant SCV's predominate (Coleman, Fischer et al. 2004). The SCV's are eventually released from host cells by active flow of fluid into the vacuole combined with periodical contractions of the host cell which result in cell rupture (Khavkin and Amosenkova 1981).

### **1.3.3 Clinical manifestation in humans**

Chronic and acute forms of Q fever are probably misdiagnosed and underreported because of the non-specific nature of Q fever symptoms (Williams and Sanchez 1994). It generally takes 7-40 days for acute Q fever to become apparent but this is dependent upon the dose, age of the individual and the route of infection (Williams and Sanchez 1994). However, due to the cryptic nature of infections with *C. burnetii* approximately 60% of cases result in asymptomatic seroconversion (Arricau-Bouvery and Rodolakis 2005). The most common symptoms associated with acute Q fever are fever, headaches, myalgia, arthralgia and rashes (Williams and Sanchez 1994). Infection with *C. burnetii* may result in endocarditis, hepatitis, pneumonia and less commonly pericarditis and myocarditis (Williams and Sanchez 1994). In general, young immunocompetent individuals tend to present with hepatitis whilst older patients are more likely to display pulmonary involvement (Fournier, Marrie et al. 1998). In addition, patients with neither hepatitis nor pulmonary involvement tend to be female (Fournier, Marrie et al. 1998). Stein and Raoult (1998) found that Q fever was a significant cause of morbidity and mortality in pregnant women in France. They found evidence of Q fever was present in at least one pregnancy per 540 and that it was the most significant public health problem related to intrauterine infections in the town of Martigues (Stein and Raoult 1998). Little is known about the symptoms and outcomes of Q fever in children (Barralet and Parker 2004). Death is a rare result

of acute Q fever and tends to be associated with chronic disease such as endocarditis in older, immunocompromised patients (Raoult, Tissot-Dupont et al. 2000).

Chronic Q fever has been defined as an infection that lasts for longer than six months after the onset and typically occurs in less than five percent of cases (Raoult and Marrie 1995). This form of Q fever may not develop for years after the primary infection and often targets the heart of the infected individual (Fournier, Marrie et al. 1998). *Coxiella burnetii* is probably the most common cause of infective endocarditis with negative blood cultures (Delahaye, Hoen et al. 2002). Clinical features of Q fever endocarditis include aortic and/or mitral valve signs, fever, cardiac failure, hepatomegaly, splenomegaly, digital clubbing, purpuric rash, and/or arterial embolism (Williams and Sanchez 1994).

#### **1.3.4 Clinical manifestation in animals**

Animal coxiellosis is usually asymptomatic with no particular tissue predilection other than the female reproductive system unless an excessive dose of *C. burnetii* is given (Williams and Sanchez 1994). Coxiellosis is an important cause of bovine reproductive disorders such as infertility, metritis and mastitis (Williams and Sanchez 1994). Reproductive disorders, including abortion, may occur in naïve animals (Arricau Bouvery, Souriau et al. 2003; Arricau-Bouvery, Souriau et al. 2005; Sanchez, Souriau et al. 2006), although in areas of high seroprevalence abortion is less common (Masala, Porcu et al. 2004). A study in Japan showed that 58.8% and 60.4% of 207 cattle with reproductive disorders had circulating antibodies to phase I and phase II *C. burnetii* respectively (To, Htwe et al. 1998). Infection of pregnant mice with *C. burnetii* normally results in uncontrolled growth of the organism in the utero-placental unit and lesions associated with the infection

are characterised by necrosis of placental tissues and microthrombosis. However, despite infiltration of the placenta by *Coxiella* no antigen has been demonstrated in foetal tissue (Baumgartner and Bachmann 1992).

A recent study in Canada found that cats which were serologically-positive for *C. burnetii* had an association with human cases of Q fever and had high phase I antibodies compared to serologically-positive cats that were not associated with human cases of Q fever (Marrie, Durant et al. 1988). In animals, unlike humans, antibodies to phase I antigen predominate in acute infections (Fournier, Marrie et al. 1998). Therefore it appears as though individuals with acute Q fever are more likely to transmit infection compared to individuals with chronic infections.

## **1.4 Epidemiology**

### **1.4.1 Transmission**

It has been demonstrated in a guinea pig model that the infectious dose of phase I *C. burnetii* may be as low as one organism (Tigertt, Benenson et al. 1961; Ormsbee, Peacock et al. 1978), which may be similar in man (Tigertt, Benenson et al. 1961). A probabilistic model developed on the assumption that a single *C. burnetii* is sufficient for infection of humans via inhalation showed that a minimum of 200 cells/m<sup>3</sup> of air was required to establish an infection (Vorobeychikov, Vasilenko et al. 2005). It has also been demonstrated that the length of the incubation period following challenge by *Coxiella* is inversely proportional to the initial infectious dose (Tigertt, Benenson et al. 1961).

Domestic ruminants appear to be the most important source of human infection (Marrie and Raoult 1997, Maurin and Raoult 1999) with inhalation of contaminated fomites via the aerosol route being the most common mode of

transmission (De Lay, Lennette et al. 1950; Abinanti, Welsh et al. 1955; Tigertt, Benenson et al. 1961; Riemann, Brant et al. 1975; Yanase, Muramatsu et al. 1998; Gardon, Heraud et al. 2001; Schulz, Runge et al. 2005). However, many other animals have been implicated in the transmission of Q fever including; kangaroos (Pope, Scott et al. 1960), feral and domestic pigs (Adesiyun and Cazabon 1996; Barralet and Parker 2004) and camels (Soliman, Botros et al. 1992),. *Coxiella* is most commonly isolated from parturient fluid and placental tissue (Baumgartner and Bachmann 1992; Masala, Porcu et al. 2004; Berri, Crochet et al. 2005) and placental tissue has been shown to have as many as  $10^9$  *Coxiella* cells per gram (Arricau-Bouvery, Souriau et al. 2005). Products of parturition can pose a significant infection risk for humans when fresh (Enright, Franti et al. 1971) and possibly more so when they dry because the coxiellae may be spread by wind (Tissot-Dupont et al 1999). However, in external environments wind does not always seem to play a significant role in dissemination (Gardon, Heraud et al. 2001; Porten, Rissland et al. 2006) and a proximity of less than six meters in enclosed spaces has been shown to be important for transmission (Porten, Rissland et al. 2006). Due to the high concentration of bacteria in products of parturition a single infected animal may be the source of infection for large numbers of people (Porten, Rissland et al. 2006).

*Coxiella burnetii* has also been shown to be shed in milk, vaginal secretions and faeces of infected animals (Berri, Souriau et al. 2002; Arricau-Bouvery, Souriau et al. 2003; Arricau-Bouvery, Souriau et al. 2005; Guatteo, Beaudeau et al. 2006). Indeed, there is evidence that goats and cows preferentially shed in milk while ewes are more likely to shed in faeces or vaginal mucus (Rodolakis, Berri et al. 2007). However, in a group of experimentally infected goats *Coxiella* was detectable by PCR in mammary gland and lung but not in liver or spleen eight days after the animals aborted (Sanchez, Souriau et al. 2006). Coxiellae were still being

shed in vaginal secretions and milk 26 and 30 days post abortion respectively (Sanchez, Souriau et al. 2006). This suggests that during pregnancy *Coxiella* may proliferate and invade several tissue types but following parturition the pathogen remains in certain tissues that are conducive to its dissemination.

Ingestion of raw dairy products has been implicated in the transmission of Q fever (Babudieri 1959; Tylewska-Wierzbanowska, Rumin et al. 1991) although the actual level of risk from this form of transmission is debatable (Cerf and Condron 2006). However, Berri et al. (2001) found that in a small flock of sheep 25% of infected ewes shed *C. burnetii* in their milk indicating that raw dairy products could pose a real risk for human infection (Berri, Souriau et al. 2002). A study in experimentally infected pregnant goats also found that *Coxiella* was shed in milk from the day of abortion until 52 days after abortion (Arricau-Bouvery, Souriau et al. 2003). In addition, *Coxiella* remain infectious in milk for up to 42 months when it is stored at 4-6°C, although heating to 63°C for 30 minutes or 72°C for 15 seconds inactivated the pathogen. Indeed, these data were used for the basis of modern pasteurisation methods (Cerf and Condron 2006).

*Coxiella* is resistant to drying and can remain infectious for long periods of time in the environment (PHAC 2001). The combination of the long term persistence of *C. burnetii* in the environment and the organism's transmission by aerosolised cells can result in cases of Q fever in individuals who have had no direct contact with livestock and therefore may confound diagnosis (Berri, Crochet et al. 2005).

A key feature differentiating *C. burnetii* from members of the genus *Rickettsia* is that *Coxiella* does not require an arthropod vector for transmission (Woldehiwet 2004). Arthropods have been shown to harbour *C. burnetii* (Smith 1940; Pope,

Scott et al. 1960) in very high concentrations (Babudieri 1959) and ectoparasites have been demonstrated to be capable of transmitting this pathogen (Arricau-Bouvery and Rodolakis 2005). However, arthropods are not considered to be a significant vector for transmission of *Coxiella* to humans (Ransom and Huebner 1951). Vertical transmission of *Coxiella* has been reported in ticks (Aitken, Bogel et al. 1987; Reeves, Loftis et al. 2005), which may enable maintenance of this pathogen in the absence of a vertebrate host.

Q fever has been shown to be a sexually transmissible disease in humans (Milazzo, Hall et al. 2001) and possibly in cattle (Tylewska-Wierzbanska, Rumin et al. 1991; Kruszewska and Tylewska-Wierzbanska 1997) and mice (Tylewska-Wierzbanska and Kruszewska 1990). Human to human transmission has been reported through placental transmission (Stein and Raoult 1998), blood transfusions (Carrieri, Tissot-Dupont et al. 2002) and during autopsies (Marmion and Stoker 1950). Trans-placental transmission was also reported in three generations of mice where the first generation was vaccinated with attenuated M-44 strain *C. burnetii* (Freylikhman et al 2003). However, there is evidence that indicates that transmission of *Coxiella* may not occur via this mechanism in sheep (Berri, Crochet et al. 2005).

#### **1.4.2 Reservoirs of infection**

*Coxiella burnetii* has been shown to be capable of infecting such a wide range of vertebrates (Marrie 1990) that it may be considered to be a pathogen with no host specificity. However, humans are dead-end hosts with regard to maintenance of the lifecycle of *C. burnetii* (Williams and Sanchez 1994). While *C. burnetii* has also been found to be able to infect a great number and variety of arthropods (Babudieri 1959; Marrie 1990) the role they play in the lifecycle of *Coxiella* is more

likely to be related to maintaining this pathogen in the environment rather than suggesting that it is primarily a vector-borne infection. *Haemophysalis humerosa* and *Amblyomma triguttatum* ticks have been implicated in the maintenance and transmission of *C. burnetii* in Australia (Derrick 1939; Smith 1940; Pope, Scott et al. 1960). The geographic range of *A. triguttatum* has recently expanded (McDiarmid, Petney et al. 2000), which suggests that the incidence of Q fever may also expand accordingly.

Domestic ruminants have historically been considered to be the primary reservoir for *C. burnetii*. However, several studies have failed to show significant evidence of infection in common livestock species (Dane and Beech 1955; Adesiyun and Cazabon 1996; Gardon, Heraud et al. 2001). It is possible that host predilection may have as much to do with geography and regional animal husbandry practices as it does with biology (Soliman, Botros et al. 1992).

The interaction between wild and domestic cycles also appears to vary according to location. A study in California, USA indicated that there was no association between the presence of *C. burnetii* in wildlife and the seropositivity of sheep (Enright, Franti et al. 1971). Conversely the same authors found that 17 of the 21 animal species tested, including coyotes, foxes, brush rabbits and deer, showed evidence of exposure to *Coxiella burnetii* (Enright, Franti et al. 1971). Indeed, there was a strong relationship between the seroprevalence in wild animals and their level of interaction with domestic livestock or small mammals (Enright, Franti et al. 1971). An epidemiological study conducted in Cyprus found that the seroprevalence of *C. burnetii* in the human population was greater than the seroprevalence in the local ruminant population (Psaroulaki, Hadjichristodoulou et al. 2006). The authors concluded that there could be another significant, as yet unidentified, reservoir of *C. burnetii* in Cyprus (Psaroulaki, Hadjichristodoulou et al.

2006). In South Australia, Dane and Beech (1955) reported that with the exception of one small mob of sheep, they were unable to find evidence that Q fever was endemic in domestic animals. The authors postulated that kangaroos may be an important permanent reservoir of Q fever and that the kangaroo tick may provide the vector for transmission of *C. burnetii* between kangaroos and livestock (Dane and Beech 1955).

Native Australian marsupials were among the first non-domestic animals shown to be infected with *C. burnetii*. *Coxiella burnetii* was isolated from bandicoots (*Isodon torosus* Ramsay) and the ticks infesting them (*Haemophysalis humerosa*) from Moreton Island (QLD) (Smith 1940). Experiments conducted on Moreton Island a year earlier revealed that bandicoots were susceptible to infection with *C. burnetii* with no apparent clinical signs (Derrick 1939). Furthermore, emulsions of ectoparasites removed from infected bandicoots failed to produce Q fever when used to inoculate naïve animals (Derrick 1939). The authors thus proposed that the carnivorous nature of the bandicoots may be important to their acquisition of *C. burnetii* (Derrick 1939).

Kangaroos and ornate kangaroo ticks in western Queensland have also been implicated as reservoirs of the causative agent of Q fever (Pope, Scott et al. 1960). Pope et al. (1960) found that in some locations the percentage of complement fixing antibodies in red kangaroos was as high as 54%. Interestingly, four of the 13 ticks from which *C. burnetii* was isolated were collected from goats and sheep and not kangaroos (Pope, Scott et al. 1960). *Amblyomma triguttatum* is a 3-host tick and thus may be able to act as a vector between the different host species. However, there has been insufficient research to accurately assess the role that Australian marsupials and their ectoparasites play in maintenance and dissemination of *C. burnetii* in Australia (Munday 1972).



### 1.4.3 Geographic distribution

In 1990 a review of literature published since 1956 noted that Q fever exists in over 70 countries covering all continents (Marrie 1990). The only region considered to be free from *C. burnetii* is the Antarctic (Woldehiwet 2004) and possibly New Zealand (Greenslade, Beasley et al. 2003). However, while Q fever is thought to be more prevalent in tropical climates (Woldehiwet 2004) the exact extent of its distribution is not known. This is in part due to misdiagnosis (Arricau-Bouvery and Rodolakis 2005) and under-reporting leading to an underestimation of the true global prevalence of this organism (Fournier, Marrie et al. 1998; Raoult, Tissot-Dupont et al. 2000). It appears that exposure to *C. burnetii* is common in all areas where specific surveillance has been performed (Arricau-Bouvery and Rodolakis 2005).

Within Australia southern Queensland and northern New South Wales have the highest number of Q fever cases in humans compared to other states with the lowest levels observed in the Northern (Garner, Longbottom et al. 1997).

### 1.4.4 Incidence and seasonal variation

There is evidence indicating that the incidence of human exposure to *C. burnetii* is increasing. During a 13 year period in Leszno, Poland (1973-1985) all 28,066 cattle tested for *C. burnetii* antibodies were found to be negative and that further surveys have shown that the number of seropositive cattle in the same areas increased to 8.4% in 1987 and 21.6% by 1989 (Tylewska-Wierzbanowska, Rumin et al. 1991). In 1988 a sero-survey performed on workers associated with livestock in the area found that 32.1% were also seropositive for *Coxiella* (Tylewska-Wierzbanowska, Rumin et al. 1991). In addition, the sero-prevalence of *C. burnetii* in humans increased from 3.1% in 1997 to 23.5% in 1999-2000 in

Newfoundland (Hatchette, Campbell et al. 2002). The incidence of clinical cases of Q fever in Germany has also increased over the period from 1947 to 1999 (Hellenbrand, Breuer et al. 2001). Hellenbrand et al. (2001) proposed that urbanisation of rural areas may be contributing to the increase in Q fever. Indeed a study in France suggests that the incidence of Q fever is underestimated based on the correlation of results of testing over 74,000 human sera for anti-*C. burnetii* antibodies with clinical findings (Raoult, Tissot-Dupont et al. 2000). However, it must also be considered that improved testing regimes and increased awareness of medical practitioners may be contributing to the trend of the increasing incidence of Q fever.

Reports of seasonal trends associated with the incidence of Q fever vary depending on geographic location. In Japan it was reported that the incidence of bovine coxiellosis increased in winter (Yanase, Muramatsu et al. 1997) while in Germany the seasonal variation of Q fever has changed from winter outbreaks to summer outbreaks (Hellenbrand, Breuer et al. 2001). In sheep in California (Enright, Franti et al. 1971) and humans in France (Raoult, Tissot-Dupont et al. 2000) and other parts of Europe (Fournier, Marrie et al. 1998) the peak incidence has been observed in spring or summer. However, in Australia two independent studies found no relationship between season and an increased incidence of Q fever (McKelvie 1980; Garner, Longbottom et al. 1997). In French Guiana it was found that over a four year period incidence of Q fever was strongly correlated with rainfall rather than season (Gardon, Heraud et al. 2001).

#### **1.4.5 Risk factors**

Q fever remains mainly an occupational hazard for people who work with domestic ruminants. There is a strong association seen in Australia between

notifications of Q fever and the presence of livestock in the same area (Garner, Longbottom et al. 1997). This conclusion supports an earlier study which examined 174 human sera for the presence of complement fixing antibodies against *C. burnetii* and found that all positive samples came from individuals that either worked in an abattoir or who worked with livestock (Dane and Beech 1955). However, a retrospective study of abattoir workers in Queensland, Australia over a nine year period showed an average incidence of 1% (McKelvie 1980). In contrast, workers at an abattoir in Brazil had a seroprevalence of 29% overall but those individuals working in areas that promoted exposure to dust and hides had a prevalence of exposure of 40% (Riemann, Brant et al. 1975).

A retrospective survey that used over 74,000 serum samples in France found that the age group most at risk of contracting Q fever was 30-69 years (Raoult, Tissot-Dupont et al. 2000). The same study showed that the disease was more prevalent in males than females but when grouped according to sex there was no statistically significant association between disease and age. The most at risk occupations for contracting acute Q fever included rural existence and contact with newborn or pregnant animals (Raoult, Tissot-Dupont et al. 2000). In addition, 23.3% of participants who had eaten farm goat cheese were sero-positive for acute Q fever (as evidenced by the presence of a higher proportion of anti-phase II antibodies compared to anti-phase I antibodies) (Raoult, Tissot-Dupont et al. 2000).

A rural existence, or an occupation that results in exposure to rural environments, were identified as significant risk factors in Germany (Hellenbrand, Breuer et al. 2001; Sting, Breitling et al. 2004; Schulz, Runge et al. 2005) and the U.S.A. (Anderson, Smoak et al. 2005).

## 1.5 Diagnosis/tests

### 1.5.1 Immunological

Diagnosis of acute Q fever is based on clinical symptoms and a fourfold rise in anti-*C. burnetii* antibodies using one of several immunological tests (Williams and Sanchez 1994). Several authors have recently suggested that the preferred test is the IFA (Peacock, Philip et al. 1983; Fournier, Marrie et al. 1998). In humans, diagnosis of chronic Q fever is made after observation of clinical syndromes and a higher proportion of anti-phase I antibodies compared to anti-phase II antibodies (Wilson, Neilson et al. 1976; Hunt, Field et al. 1983; Peacock, Philip et al. 1983; Tissot-Dupont, Thirion et al. 1994). In livestock, *C. burnetii* infection can be diagnosed at the herd level by a combination of serological tests, clinical findings and microscopy of stained placental smears (OIE 2000).

Effective immunological diagnosis of Q fever should be made after testing multiple serum samples with a test capable of detecting anti-phase I and anti-phase II antibodies (Dupuis, Peter et al. 1985). It has been shown that anti-phase II antibodies can persist in humans for long periods of time whereas anti-phase I antibodies begin to decline after 12 weeks (Dupuis, Peter et al. 1985). Therefore testing for only anti-phase I antibodies may lead to a false negative result and a test which is solely focused on anti-phase II antibodies may indicate a positive in an individual who has already cleared the infection.

Many immunological tests for *Coxiella* have been developed with varying levels of specificity and sensitivity. In general the immunological tests used for detecting exposure to *C. burnetii* fall into three categories: complement fixation, indirect immunofluorescence, and ELISA.

### 1.5.1.1 Complement-fixation test (CFT)

Complement-fixation is very specific but lacks sensitivity (Peter, Dupuis et al. 1985). However it has the advantage that it can be adapted to test for exposure to *C. burnetii* without the requirement for host species-specific antibodies (Peter, Dupuis et al. 1985). Correct interpretation of results in humans requires both convalescent and acute phase samples and diagnosis can be confounded by the nature of complement fixing antibodies which are often not present early in infection but may persist for long periods after the primary illness (Peter, Dupuis et al. 1985). In general it has been shown that phase I *C. burnetii* fixes complement much more weakly than does phase II (Enright, Longhurst et al. 1971). It has also been found that the sensitivity (73%) and specificity (90%) of CFT is inferior to the same measures in an ELISA (99% and 88% respectively) when IFAT was used as the reference method (Field, Mitchell et al. 2000).

### 1.5.1.2 Indirect immunofluorescence assay (IFA)

The IFA is currently the method of choice for detection of anti-*C. burnetii* antibodies (Peacock, Philip et al. 1983; Field, Mitchell et al. 2000; OIE 2000). IFA is superior to the CFT (Ascher, Greenwood et al. 1981; Dupuis, Peter et al. 1985; Peter, Dupuis et al. 1985; Peter, Dupuis et al. 1987) and can be used to detect antibodies to phase I and II antigens in the IgG, IgM and IgA fractions (Fournier, Marrie et al. 1998). Dupont et al. (1994) found that in the microimmunofluorescence test phase I IgA titres are not predictive of chronic Q fever whereas phase I IgG is highly predictive and sensitive. The same group also found that while diagnosis of chronic Q fever can be made using phase II IgG and IgA titres the sensitivity is very low (57%) (Tissot-Dupont, Thirion et al. 1994). Setiyono et al. (2005) stated that the IFA should be used with an “equivocal area” rather than a single cutoff value and that any sera in the equivocal area should be

tested with a secondary immunological assay to eliminate false positive and false negative results (Setiyono, Ogawa et al. 2005). Diagnosis by this method may also be confounded by non-specific reactions with rheumatoid factor, therefore an adsorbent is required to remove IgG prior to testing for anti-*C. burnetii* IgM and IgA (Fournier, Marrie et al. 1998). This method has also been assessed for cross-reactivity with *Legionella*, one of the closest relatives of *C. burnetii*. Legionellosis and Q fever may both present with identical symptoms but require different antimicrobial therapies to treat the infection. Therefore correct diagnosis is of the utmost importance. A study in France showed that there was no cross-reactivity between antibodies to *Coxiella* and *Legionella* in clinically affected individuals detected using IFA (Finidori, Raoult et al. 1992). However, two of the major criticisms of the IFA are that it has a subjective component and it is not easily standardised (Peter, Dupuis et al. 1988; Field, Mitchell et al. 2000).

#### **1.5.1.3 Enzyme-linked immunosorbent assay (ELISA)**

Several studies have found that the ELISA is superior to both the CFT and IFA (Peter, Dupuis et al. 1985; Peter, Dupuis et al. 1987; Soliman, Botros et al. 1992). Other studies have also suggested that it should be considered as a useful aid to diagnosis by IFA (Field, Mitchell et al. 2000) or perhaps has application as an epidemiological tool (Peter, Dupuis et al. 1987). The increased sensitivity that is achievable with ELISA is attractive but some consider that its diagnostic utility is limited by the technical proficiency required to conduct the test and interpret the results (Fournier, Marrie et al. 1998).

Indirect ELISA, like IFA, is also limited in that species-specific antibodies are required for a particular application. However, alternatives are available. Soliman et al. (1992) developed a competitive ELISA to detect *C. burnetii* in camels

because anti-camel immunoglobulin is not commercially available. The competitive ELISA was shown to detect more positives than IFA or EIA in all animal sera used in this study (Soliman, Botros et al. 1992). Field and company (2000) compared a commercial human IgM Q fever ELISA manufactured by PanBio (Australia) with the indirect fluorescent antibody test (IFAT) for anti-*C. burnetii* IgM and the complement fixation test (CFT). Some cross-reactivity was seen in the ELISA with patients that had rickettsial infections (two of five patients) and false positives were recorded for two out of five sera from patients with leptospirosis and one out of four samples containing rheumatoid factor (Field, Mitchell et al. 2000).

A critical component of all antibody-detection ELISA's is the antigen. The sensitivity and specificity of antigens made from *C. burnetii* whole cells can vary considerably (Dane and Beech 1955; Kovacova, Kazar et al. 1998). In addition, the validity of using Nine Mile strain *C. burnetii* in antigen preparations has been questioned (Rodolakis, Bouzid et al. 2007). Production of an antigen from live organisms is made difficult and expensive by the high level of biosecurity required to work with *C. burnetii*. Thus, production of a purified antigen which is cheap, safe and applicable for a broad geographic range may well be impossible and there is a pressing need for a recombinant antigen that can overcome these issues. Currently, recombinant proteins that can serve as antigens have displayed only moderate immunogenicity (Zhang and Samuel 2003; Tyczka, Eberling et al. 2005). Although the use of two *C. burnetii* fusion proteins appears to produce a more pronounced immune response (Li, Niu et al. 2005).

### 1.5.2 Polymerase chain reaction (PCR)

Assays based upon PCR technology require careful design to ensure they produce meaningful results. Of particular importance is selection of the gene sequence that the test will target. Targets previously used for the detection of *C. burnetii* include the outer membrane protein gene *com1* (Zhang, Nguyen et al. 1998; Brennan and Samuel 2003), the superoxide dismutase gene (Stein 1992; Masala, Porcu et al. 2004) and the *IS1111a* insertion sequence (Berri, Laroucau et al. 2000; Klee, Tyczka et al. 2006). The *IS1111a* element makes an attractive target due to its high copy number and lack of significant homology to published sequences from other related organisms (Hoover, Vodkin et al. 1992).

One disadvantage of PCR however, is that environmental matrices that have high organic content such as faeces and soil often contain factors that are considered to be inhibitory to PCR (REF). To minimise the confounding effects of tannins, humic acid and other inhibitors of PCR it is critical to extract high quality DNA from the sample while removing the majority of contaminants (Jiang, Alderisio et al. 2005). Current DNA purification techniques are not capable of removing all confounding factors from varied and complex samples such as faeces and soil. To ameliorate the affect of such factors facilitators of the PCR can be added prior to amplification. The most effective PCR facilitators include bovine serum albumin and T4 gene 32 protein (Jiang, Alderisio et al. 2005).

PCR-based assays to detect *C. burnetii* have been developed and applied on a herd or flock basis. Guatteo et al. (2006) showed that 65% of dairy cattle from a herd of 242 had *C. burnetii* in milk, vaginal mucus or faeces using real-time PCR. The identification of *C. burnetii* in only one sample from each animal suggested that a prudent policy requires collection and testing of multiple types of samples



from each animal to enable consistent detection of animals which are shedding *Coxiella* (Guatteo, Beaudeau et al. 2006).

PCR-based assays have also been used to detect *Coxiella* paraffin-embedded samples from humans with chronic endocarditis of unknown aetiology and enable a retrospective diagnosis of chronic Q fever (Yuasa, Yoshiie et al. 1996). PCR has also been used for the direct identification of *C. burnetii* plasmids in human sera (Zhang, Hotta et al. 1998). If plasmid type can truly be correlated with disease outcome, this test would allow more rapid administration of chemotherapy which is appropriate to the chronic or acute form of the infection. Zhang et al. also published a PCR targeting the *com1* gene which they proposed could be used diagnostically because it may allow diagnosis in the early stages of infection where a detectable immune response is not present (Zhang, Nguyen et al. 1998). However, the transient nature of Q fever bacteraemia (Williams and Sanchez 1994) may limit the usefulness of this test to early acute infections.

Further development of the basic principle of PCR has seen the advent of techniques that can estimate DNA concentration in the starting sample (Zhang, Wen et al. 2005; Klee, Tyczka et al. 2006), improve sensitivity by hybridisation with other technologies (Muramatsu, Yanase et al. 1997), determine antibiotic and disinfectant susceptibility of obligate intracellular bacteria (MacDonald, Sargent et al. 2002; Brennan and Samuel 2003) and enable gene expression and viability experiments (Fontaine and Guillot 2003; Coleman, Fischer et al. 2004).

## 1.6 Viability

The perceived need for bacterial viability assays, partly due to increased awareness of the potential of bioterrorism, has led to the development of many new techniques. Fluorescence assays in particular have received attention with

the most common tests targeting respiratory activity (Bhupathiraju, Hernandez et al. 1999), membrane potential (Deere, Porter et al. 1995) and membrane integrity (Nebe-von-Caron, Stephens et al. 2000). Laflamme et al. (Laflamme, Lavigne et al. 2004) consider that all of these assays must be performed to adequately assess viability. However, performing all of these tests is both time consuming and expensive. Therefore, more unequivocal tests are required for routine monitoring and surveillance applications. Chemicals that pass through compromised cell membranes and render DNA unavailable to replicative processes have also been used, in combination with quantitative PCR, to estimate viability (Rudi, Moen et al. 2005; Rueckert, Ronimus et al. 2005).

### **1.6.1 Animal inoculation**

Animal models have been used to assess the efficacy of antibiotics against *C. burnetii* (Raoult 1993) and they remain the gold standard for estimating the viability and infectivity of this pathogen (Arricau-Bouvery, Souriau et al. 2005). Scott and Williams (1990) used an adaptation of an earlier method (Ransom and Huebner 1951) consisting of infection of chick embryos and sub-passage in mice and detection of an immunological response to verify the infectivity of disinfectant-treated samples. This procedure took in excess of four weeks to produce results (Scott and Williams 1990). Development of new techniques for estimating viability are vitally important to *C. burnetii* research due to the expense, technical complexity and ethical issues associated with animal assays (Ransom and Huebner 1951; Malloch and Stoker 1952; Sobsey and Leland 2001).

### **1.6.2 Culture-based methods**

Plaque assays have been successfully applied to several rickettsial species (McDade and Gerone 1970; Wike, Tallent et al. 1972; Cory, Yunker et al. 1974;

Ormsbee, Peacock et al. 1978; Schneider 1989). However, limited success has been achieved in the application of these techniques to *C. burnetii* (Kordova 1966; Cory, Yunker et al. 1974; Schneider 1989). Indeed, the results that were obtained correlated poorly with titrations in animals and took longer than two weeks to complete (McDade and Gerone 1970; Wike, Tallent et al. 1972). An adaptation of the plaque assay has been described, which enumerated stained vacuoles in vero cells rather than plaques in a confluent monolayer (Schneider 1989). The authors proposed that this method was more rapid and economical and less laborious than previously described plaque formation assays.

While an effort to grow *C. burnetii* in axenic media was unsuccessful the study did show that significant biosynthetic processes occurred in the pathogen when grown in a cell-free acidic medium (Zuerner and Thompson 1983). These findings support an earlier study which demonstrated that the subcellular machinery of *C. burnetii* was able to synthesis coliphage protein *in vitro* when supplied with amino acids and mRNA (Donahue and Thompson 1980; Donahue and Thompson 1981). Furthermore, Hackstadt and Williams found that in acidic axenic medium *C. burnetii* produced RNA and DNA when supplied with an energy source and that glutamate supplied to the organism was incorporated into a trichloroacetic acid insoluble product. These results indicate a functional protein synthetic mechanism exists in *Coxiella* (Hackstadt and Williams 1981) and that culture of this organism in axenic medium may be possible.

Brennan and Samuel (2003) described a cell culture-based method for evaluating disinfection efficacy, a task made difficult by the intracellular growth requirements of *C. burnetii*. This type of assay has been used successfully for growth, viability and disinfection assays for *Cryptosporidium parvum* (Shin, Linden et al. 2001; MacDonald, Sargent et al. 2002; Rochelle, Marshall et al. 2002;

Keegan, Fanok et al. 2003). The shell-vial assay has also proven to be effective in this type of application (Raoult 1993).

### **1.6.3 Molecular methods**

Detection of transcriptional activity is considered to be indicative of viability (Keer and Birch 2003). Reverse transcriptase PCR is not a new technique but advances in quantitative estimation of nucleic acids has exponentially increased the power of this type of assay (Bustin and Nolan 2004). Application of RT-qPCR has helped to elucidate the developmental cycle of *C. burnetii* (Coleman, Fischer et al. 2004) and has been used to estimate the viability of other organisms (Jenkins, Trout et al. 2003; Keer and Birch 2003; Ohashi, Inaoka et al. 2003). However, while the transient nature of RNA makes it an ideal target for viability assays, such techniques require careful planning and a thorough knowledge of the potential pitfalls of the technology before they can be applied to real samples (Jenkins, Trout et al. 2003; Bustin and Nolan 2004; Huggett, Dheda et al. 2005).

### **1.6.4 Membrane integrity and membrane potential-based methods**

The assumption that dead cells lose their membrane integrity has been the foundation for several methods of viability assessment. Viable cells control the trans-membrane traffic of solutes and therefore any indication that movement across the cell membrane is not regulated is a proxy measure of viability (Laflamme, Lavigne et al. 2004). Ethidium monoazide bromide (EMA) is a nucleic acid binding dye which, once photolysed, covalently links to DNA thus preventing amplification by PCR (Waring 1965). It has been proposed that EMA selectively crosses the membranes of dead cells but is unable to penetrate live cells (Rudi, Moen et al. 2005) thus providing a useful tool for determining viability. However, this method may not be broadly applicable because it has been shown to be toxic

to some organisms and may also cross the membranes of live cells (Rueckert, Ronimus et al. 2005). Other viability assays have also been developed which take advantage of the correlation between cell viability and membrane integrity. The commercially available Live/Dead *BacLight* kit enables visualisation of viable/non-viable cell populations under UV illumination following the penetration of the DNA-binding dye SYTO9 through compromised cell membranes (Laflamme, Lavigne et al. 2004). However, this method is limited by the resolution of a light microscope, thus excluding small cells. Like EMA, the DNA-degrading enzyme DNase selectively crosses the membranes of dead cells only and subsequently degrades the DNA within (Frankfurt 1983), a property which has been exploited in PCR and flow cytometric viability assays (Frankfurt 1983; Rueckert, Ronimus et al. 2005).

## **1.7 Treatment and control of Q fever infections**

### **1.7.1 Antibiotic therapy**

The acute form of Q fever is normally diagnosed retrospectively because it is a febrile self-limiting illness with non-specific symptoms (Williams and Sanchez 1994). However, recovery can be accelerated by treatment with tetracyclines or a variety of other antibiotics (Woldehiwet 2004) if the intervention is initiated in the first three days of infection (Raoult 1993). Chronic Q fever, on the other hand, can be much more difficult to treat as it is refractory to antibiotics and has been shown cause mortality in approximately 2% of cases (Raoult 1993) in some instances mortality may result even when appropriate antibiotic treatment is administered (Williams and Sanchez 1994). Currently the recommended treatment for chronic Q fever is doxycycline plus hydroxychloroquine for a minimum of 18 months (Raoult, Houpikian et al. 1999).

### 1.7.2 Vaccination

Several vaccine formulations are available, with the most common consisting of formalin-inactivated whole cell preparations (Waag, England et al. 1997). Despite the simple nature of this type of vaccine it has proven to be extremely effective at preventing Q fever (Ackland, Worswick et al. 1994). In an effort to reduce the incidence of Q fever the Australian government implemented the National Q Fever Management Program in 2000. This program aimed to reduce the burden of disease associated with Q fever and included subsidies for vaccination of at-risk workers (Kermode, Yong et al. 2003). It was estimated using data from 1993-94 that Q fever in Australia may cost the country A\$1 million per year (Garner, Longbottom et al. 1997). Therefore it is not surprising that an economic evaluation of the impact of Q fever on workers in the meat and agricultural industries in Australia found that increasing the rate of vaccination yielded significant financial benefits to employers. It was suggested that increasing Q fever vaccination of meat industry workers would prevent 400 cases of Q fever, four deaths and result in a gain of 43 discounted life years over a 20 year period. This equates to a cost benefit of \$20,000/life year gained (Kermode, Yong et al. 2003).

The vaccine used is made from inactivated whole cell phase I Henzerling strain *C. burnetii* (Q-Vax, CSL) and appeared to be protective for at least five years, although repeated natural exposure may contribute to protection (Ackland, Worswick et al. 1994). Administration is by a single subcutaneous injection of 30 µg of vaccine following pre-vaccination antibody assay and skin test to ensure that the individual is unsensitised to *C. burnetii*. Vaccination of individuals who have previously been exposed to the Q fever agent has resulted in serious side effects such as severe necrotic lesions or granuloma development at the injection site

(Marmion, Ormsbee et al. 1984; Fairweather, O'Rourke et al. 2005). It was also demonstrated that when mice were infected with an attenuated strain of *C. burnetii* that is commonly used to make the vaccine, live organisms could be isolated not only from the vaccinated animals but also from their F1 and F2 offspring (Freylikhman, Tokarevich et al. 2003).

Fractionation of whole cell preparations has been performed in an effort to develop a new Q fever vaccine (Fries, Waag et al. 1993). The ineffectiveness or toxicity of fractionated and/or purified vaccines and the possibility that whole-cell vaccines may contain live *Coxiella* highlight the need for a recombinant alternative. Efforts to create a subunit vaccine have met with some success in mice but the response seen was still inferior to that generated by a formalin-fixed whole cell antigen of *C. burnetii* (Li, Niu et al. 2005). Similarly, investigations into applying recombinant *C. burnetii* proteins as vaccine antigens has met with limited success (Zhang, Kiss et al. 2004; Tyczka, Eberling et al. 2005).

### 1.7.3 Disinfection

*Coxiella burnetii* is highly resistant to physical and chemical stress and thus poses a significant problem for both laboratory and industrial disinfection strategies alike. Ultra violet irradiation has been shown to be effective in inactivating *C. burnetii* at relatively low doses (Ransom and Huebner 1951; Little, Kishimoto et al. 1980). However, a more recent study demonstrated that *C. burnetii* has a fully functional DNA excision repair system despite lacking some of the genetic elements normally associated with repair of oxidative damage (Mertens, Lantsheer et al. 2005). Therefore, it remains uncertain whether using UV radiation to inactivate *C. burnetii* is a valid approach for disinfecting water. The UV dose typically applied in wastewater treatment processes is in the order of 30 to 40

mJ/cm<sup>2</sup> (Shin, Linden et al. 2001), which is nearly two orders of magnitude greater than the dose that appears to be effective for inactivating *C. burnetii* (Little, Kishimoto et al. 1980). The damage induced at this level of radiation may well be too severe to be repaired by the mechanisms available to *C. burnetii*.

Scott and Williams (1990) found that liquid suspensions of *C. burnetii* exposed to 0.5% NaOCl, 2% Roccal, 5% Lysol or 5% formalin for 24 hours were still infectious. However, 30 minute exposure to 70% ethanol, 5% chloroform or 5% EnviroChem resulted in inactivation (Scott and Williams 1990). The resistance of *C. burnetii* to other chemical agents such as formalin and phenol has also been found to be superior to comparable organisms (Ransom and Huebner 1951). However, the availability of new techniques for assessing the viability of intracellular parasites may necessitate that the efficacy of some of these agents be reassessed.

Current pasteurisation methods are predicated upon their ability to inactivate indicator organisms such as *C. burnetii* (Cerf and Condron 2006). It has been demonstrated that *C. burnetii* remains viable for between 30 and 60 minutes when exposed to 60°C in a liquid suspension of cells and that 65°C for 30 minutes was sufficient to inactivate both strains tested (Ransom and Huebner 1951). The energy required to generate temperatures of this magnitude in large-scale effluent streams preclude such uses but heating is clearly an option for laboratory sanitisation.

State-of-the-art technologies such as photocatalytic treatment of air (Vohra, Goswami et al. 2005) may provide solutions in the future and while their application to agricultural situations is currently limited it may be beneficial to implement such strategies in research situations.



#### 1.7.4 Behavioural tools for Q fever control

An epidemiological investigation into an outbreak of Q fever in a small town in the French Alps in 1996 indicated that the source of the infections was a pile of contaminated uncovered sheep waste in a local abattoir. It was suggested that spread of *Coxiella* was facilitated by a nearby heliport (Carrieri, Tissot-Dupont et al. 2002). This incident highlights that good work practices and basic knowledge of how Q fever can be spread is sufficient in many instances to prevent outbreaks of the disease. Furthermore, Hellenbrand et al. (2001) proposed that with increasing urbanisation of rural areas the most effective method for preventing Q fever is to limit sheep-related exposures. In the event that contact with sheep, and indeed other ruminants, is unavoidable, crutch clipping of the ewe's wool prior to lambing and total confinement housing at lambing in winter and spring may lower the chance that a flock would be seropositive for *Coxiella* and subsequently decrease the chance of zoonotic transfer (Lang, Waltner-Toews et al. 1991). However, perhaps the most useful behavioural tool for the control of Q fever is awareness of this disease within industries associated with domestic and feral ruminants. In this way effective disinfection and, if required, vaccination programs can be implemented. Furthermore, in the event of an outbreak of Q fever, adequate awareness will enable rapid diagnosis and thus the implementation of the appropriate intervention.

## **2. The development and validation of a PCR-based detection system for *Coxiella burnetii* in environmental samples**

### **2.1 Introduction**

Q fever is a serious occupational hazard for people who work with or around livestock or marsupials including kangaroos and bandicoots. This disease is rarely fatal but it can have a significant effect on the health and productivity of infected individuals. The agent responsible for Q fever, *Coxiella burnetii*, has a strong association with domestic ruminants including sheep, goats and cattle (Raoult and Marrie 1995) as well as native Australian marsupials (Derrick 1939; Pope, Scott et al. 1960). Inhalation of contaminated fomites via the aerosol route is the most common mode of transmission (Yanase, Muramatsu et al. 1998) with as few as one viable *C. burnetii* cell capable of causing clinical disease in humans (Tigertt, Benenson et al. 1961; Ormsbee, Peacock et al. 1978). *Coxiella* can persist in the environment for an extended period because it is extremely resistant to physical and chemical damage (PHAC 2001). Therefore, environmental substrates that have been contaminated by *C. burnetii* from infected domestic, feral or wild animals can pose a risk of infection to humans for several months.

Polymerase chain reaction-based tests to detect *C. burnetii* in milk, dust, urine, faeces, genital swabs, air, clinical samples and cell culture have been published (Muramatsu, Yanase et al. 1997; Yanase, Muramatsu et al. 1998; Berri, Laroucau et al. 2000; Issartel, Gauduchon et al. 2002; Brennan and Samuel 2003; Klee, Tyczka et al. 2006). There are no reports on the validation of a PCR-based test detecting *C. burnetii* in environmental samples such as raw water, dust or faeces. These environmental substrates can confound PCR-based detection assays because they contain inhibitors that are difficult to remove such as humic

and fulvic acids and tannins (Kreader 1996; Hartman, Coyne et al. 2005; Klerks, van Bruggen et al. 2006; Whitehouse and Hottel 2007). Thus there is a need for a robust and broadly applicable PCR that has defined properties including sensitivity, specificity and repeatability.

## **2.2 Materials and methods**

### **2.2.1 DNA extraction and standard curve preparation**

Q-Vax™ (CSL, Parkville, Australia), which comprises formalin inactivated whole *C. burnetii* (phase I Henzerling strain) was used to prepare all reference DNA standards and standard curves. A suspension containing 25 µg of *C. burnetii* cells from Q-Vax™ vaccine was pelleted by centrifugation at 20,800 x *g* for 10 minutes and whole genomic DNA extracted using a Qiagen Tissue Minikit according to the manufacturers instructions (Qiagen, Hilden, Germany) except with a 45 minute incubation at 55°C in buffer ATL with proteinase K and a final 30 minute incubation at 70°C prior to the addition of 70% v/v ethanol (Klee, Tyczka et al. 2006). DNA was eluted in 100 µl of buffer AE, quantified with a Nanodrop spectrophotometer (Nanodrop Technologies Wilmington USA) and converted to genome copy number using the formula described by Brennan and Samuel (Brennan and Samuel 2003). Standard curves were prepared by making serial 10-fold dilutions of the DNA extract in TE buffer.

### **2.2.2 Primer design**

The primer and probe set named with the prefix TP1 were developed by the Australian Rickettsial Reference Laboratory (Geelong, Victoria, Australia). The primer set named with the prefixes RAF and FAF are from previously published work (Brennan and Samuel 2003) and the primer and probe sets with the prefixes

ScvA and 16S were from a published study by Coleman et al. (2004). All primers not obtained from published methods were designed using Primer3 online software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Primers were synthesised by Invitrogen (Mount Waverley, Victoria, Australia) and the oligonucleotide probes were supplied by Geneworks (Hindmarsh, South Australia). A list of the primer and probe sequences used is shown in Table 2.1.

**Table 2-1. Primer and probe sequences used to detect *Coxiella burnetii* DNA in a real-time PCR format**

<b>Name</b>	<b>Gene</b>	<b>Sequence (5' to 3')</b>	<b>Labels</b>
	<b>Targeted</b>		
<b>IS1111aF</b>	<i>IS1111a</i>	GTTTCATCCGCGGTGTTAAT	None
<b>IS1111aR</b>	<i>IS1111a</i>	TGCAAGAATACGGACTCACG	None
<b>IS1111aP</b>	<i>IS1111a</i>	CCCACCGCTTCGCTCGCTAA	5'-6FAM 3'-BHQ- 1
<b>TP1f</b>	<i>Com1</i>	AAAACCTCCGCGTTGTCTTCA	None
<b>TP1r</b>	<i>Com1</i>	GCTAATGATACTTTGGCAGCGTATTG	None
<b>TP1p</b>	<i>Com1</i>	AGAACTGCCCATTTTGGCGGCCA	5'-6FAM 3'-BHQ- 1
<b>FAF216</b>	<i>Com1</i>	GCACTATTTTGTAGCCGGAACCTT	None
<b>RAF290</b>	<i>Com1</i>	TTGAGGAGAAAACTGGATTGAGA	None

<b>HspB-F</b>	<i>HspB</i>	CTCCACGCGGTTTTTAATGT	None
<b>HspB-R</b>	<i>HspB</i>	CGGGCGGTAAGGTTATTTCT	None
<b>HspB-P</b>	<i>HspB</i>	CGGCGTCACCAGAACCATCAATG	5'-6FAM
			3'-BHQ-
			1
<b>SpoIIIE-F</b>	<i>SpoIIIE</i>	CGCTAAATCCACGATGACTG	None
<b>SpoIIIE-R</b>	<i>SpoIIIE</i>	GGAAATCGGTTATTGGTCTGG	None
<b>SpoIIIE-P</b>	<i>SpoIIIE</i>	CGCCAACACTTCGTAAATCGTGACC	5'-6FAM
			3'-BHQ-
			1
<b>ScvA-F</b>	<i>ScvA</i>	TGGAAAGACAAAATGTCCAACAA	None
<b>ScvA-R</b>	<i>ScvA</i>	GGTTAGAAGCACCCGGTCGT	None
<b>ScvA-P</b>	<i>ScvA</i>	ACGTGGAAAAGACCAACG	5'-6FAM
			3'-BHQ-
			1

<b>16S-F</b>	<i>I6S</i>	CCATGAAGTTGGAATCGCTAG	None
<b>16S-R</b>	<i>I6S</i>	ACTCCCATGGTGTGACGG	None
<b>16S-P</b>	<i>I6S</i>	CGGTGAATACGTTGCGGGCCTTGTA C	5'-6FAM  3'-BHQ- 1
<b>JB153-3-F</b>	<i>JB153-3</i>	TATTCGGCATCCCTTGGATA	None
<b>JB153-3-R</b>	<i>JB153-3</i>	TTGTAACGCGCCACTATCTG	None
<b>JB153-3-P</b>	<i>JB153-3</i>	TCACGCGCAATATTTGCAGCATG	5'-6FAM  3'-BHQ- 1

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For primers that were designed in-house whole gene sequences were submitted online to the NCBI database searching tool BLASTn (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) for homology matching. Regions with little or no homology to other submitted sequences were selected for subsequent design of oligonucleotide primers and probes. All primers and probes were then compared to all published sequences using BLASTn to identify any significant matches.

### **2.2.3 PCR optimisation**

Forward and reverse primers were titrated against each other in concentrations ranging from 5 pmol to 25 pmol in increments of 5 pmol. For assays with TaqMan probes the probe concentrations were titrated from 1.25 pmol to 5 pmol in increments of 1.25 pmol. The optimum concentration of each primer and probe combination that yielded the lowest Ct value and the largest difference between peak fluorescence and background fluorescence were selected. All samples were performed in duplicate and no template (negative) controls were included for each primer set.

TaqMan assays were optimised for magnesium chloride concentration in the range 1.5 mM to 4.5 mM in increments of 0.5 mM. Once optimum primer concentrations were established some primer sets (IS1111a, TP1 and RAF/FAF), with no probe added, were titrated for optimum SYBR<sup>®</sup> Green I (Sigma-Aldrich, Castle Hill, NSW, Australia) concentration and re-titrated for magnesium chloride in light of the requirements of this detection chemistry. Magnesium chloride concentrations ranged from 2 mM to 4.5 mM and dilutions of 1 in 25,000, 50,000, 100,000, 200,000 and 400,000 of the 10,000X SYBR<sup>®</sup> Green I stock were made. Melt curve analysis was performed on all primer sets to ensure the absence of



secondary peaks which are indicative of non-specific amplification. Annealing temperatures from 58 to 66°C in 1° increments were titrated against a set of serial 10-fold dilutions of *C. burnetii* DNA for all primer sets except the RAF/FAF set for which the published annealing temperature was used (Brennan and Samuel 2003). Where the reaction efficiency for a particular assay deviated too far from the ideal value higher annealing temperatures were not tested. Ideal annealing temperatures were selected based upon reaction efficiencies, Ct values, lack of signal in NTC controls, amplification of low concentrations of template and linearity of standard curves.

#### **2.2.4 PCR conditions**

All SYBR® Green reactions contained 0.01 µmoles of dNTP mixture (Fisher Biotech, West Perth, Western Australia, Australia) magnesium chloride, primers, 5.5 units of Tth Plus DNA Polymerase (Fisher Biotech), 10× reaction buffer, SYBR® Green I, SYBR® Green buffer made according to the protocol described previously (Karsai, Muller et al. 2002), template DNA and water to a total reaction volume of 25 µl.

TaqMan real-time PCR reaction mixtures contained 0.01 µmoles of dNTP mixture magnesium chloride, primers, probe, 5.5 units of Tth Plus DNA Polymerase, 10× reaction buffer, template DNA and water to a total reaction volume of 25 µl.

Real-time PCR assays were performed on a Rotorgene® 3000 (Corbett Life science, Mortlake, New South Wales, Australia) according to one of the two following cycling parameters:

TaqMan: one hold at 95°C for two minutes followed by 40 cycles of 95°C for 20 seconds and 40 seconds at the relevant annealing temperature.

SYBR: one hold at 95°C for two minutes followed by 40 cycles of 95°C for 20 seconds, 35 seconds at the relevant annealing temperature, 72°C for 20 seconds, a final hold for 20 seconds at 52°C leading into a melt curve analysis up to a maximum temperature of 99°C.

Following all qPCR amplification experiments the Rotorgene<sup>®</sup> 3000 software was used to automatically select optimal cycle threshold cut-offs based upon the slope of the standard curve and the  $R^2$  value. The user-defined DNA concentrations of the standards were then used by the software to fit a curve to the samples and subsequently estimate the concentration of DNA in each standard. Melt curve peaks were determined using the Rotorgene<sup>®</sup> 3000 software with all digital filters turned off to maximise the precision of oligonucleotide melt temperature estimation.

### **2.2.5 Reproducibility**

Each assay was used to amplify *C. burnetii* DNA that was prepared in seven serial 10-fold dilutions from  $5.56 \times 10^6$  genomes per micro litre down to 5.6 genomes per micro litre. All primer sets were evaluated using both SYBR<sup>®</sup> and TaqMan detection systems with the exception of FAF216/RAF290 which was only used in the SYBR<sup>®</sup> system. Samples were performed in duplicate with two NTC's included in each run. All assays were repeated six times and the data generated were analysed using the Statistical Package for Social Sciences (version 15.0, SPSS Inc., Chicago, USA) to determine means and standard errors of the means (SEM) for each dilution point.

The effect of making bulk reaction mixtures (with all components added other than DNA, SYBR<sup>®</sup> Green and primers) and storing them -80°C prior to use was assessed with the FAF216/RAF290 primer set. The cycling conditions when using this master mix were identical to all other SYBR<sup>®</sup> Green assays.

A commercial TaqMan master mix (Platinum<sup>®</sup> Quantitative PCR SuperMix-UDG, Invitrogen) was also assessed for its ability to improve reproducibility when used in conjunction with the IS1111a primer and probe set. The reaction concentrations used with this master mix were 12.5 µl of Invitrogen master mix, one micro litre of each primer and probe, 0.75 µl of 50 mM MgCL<sub>2</sub>, one micro litre of DNA template and water to 25 µl. The cycling conditions when using this master mix were identical to the other TaqMan assays other than the addition of a two minute hold step at 50°C at the beginning of the run.

### **2.2.6 Analytical sensitivity**

The analytical sensitivities of the IS1111a, TP1, HspB, JB153-3 and SpolIIE TaqMan assays were determined by testing a standard curve consisting of eight serial 10-fold dilutions where the highest dilution point had a calculated concentration of 0.16 genomes per micro litre. Each dilution was tested in duplicate, each run had two NTC's included and each assay was repeated three times. The data were analysed as described previously except the SPSS package was also used to provide descriptive statistics including mean, standard deviation, standard error of the mean and the coefficient of variation. Microsoft Excel XP Professional Edition (Microsoft Corp, California, USA) was used to graph the relationship between *C. burnetii* DNA standard concentration and cycle threshold values for all tests.

### **2.2.7 Analytical specificity**

The analytical specificities of the IS1111a, TP1, HspB, JB153-3 and SpoIIIE TaqMan assays were determined by attempting to amplify 10 ng of DNA per reaction from the organisms listed in table 2.2. All assays used the same reaction mixtures and cycling conditions as before.

**Table 2-2. Sources of organisms used to assess the specificity of qPCR assays**

<b>Organism</b>	<b>Supplier</b>	<b>Source</b>
<i>Staphylococcus aureus</i>	MU	ARC mouse lesion
<i>Streptococcus equi</i>	AgWA	Case No 196/81 Equine Nasal
<i>Pseudomonas aeruginosa</i>	MU	Skin swab, general dermatitis
<i>Yersinia enterocolitica</i>	AgWA	Case No AS-01-2175 #4
<i>Yersinia pseudotuberculosis</i>	AgWA	Case No PR 3696/84 Porcine
<i>Brucella abortus</i>	AgWA	
<i>Brucella ovis</i>	AgWA	Case No AS-06-2900 Ovine
<i>Klebsiella oxytoca</i>	AgWA	Case No P-00-1599 Yabby Tissue
<i>Serratia marcescens</i>	AgWA	Case No PR1584/81 Ovine Duodenum
<i>Proteus mirabilis</i>	AgWA	Case No AS-06-2916 Murine Nasal
<i>Legionella pneumophila</i>	Ecovise	
<i>Rickettsia rickettsii</i>	ARRL	

*Rickettsia prowazekii*

ARRL

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MU = Murdoch University

DAFWA = Department of Agriculture and Food, Western Australia

ARRL = Australian Rickettsial Reference Laboratory

### 2.2.8 Culture, extraction and purification of *Coxiella burnetii*

*C. burnetii* phase II Nine Mile clone 4 originally isolated from a tick from Montana, USA in 1935 (Denison, Massung et al. 2007) was kindly supplied by Professor Jim Samuel from the Department of Intracellular Pathogens, Faculty of Medicine, Texas A&M University. Confluent monolayers of Vero (Green monkey kidney) cells were infected by adding 2 µl of 3 mg/ml *C. burnetii* phase II to each 25 cm<sup>2</sup> culture flask (TPP, Switzerland). Cells were maintained in DMEM (MP Biomedicals, Australia) in an atmosphere containing 5% (vol/vol) CO<sub>2</sub> supplemented with 200mM L-Glutamine (MP Biomedicals, Australia) and 2% v/v foetal calf serum. Media was not replenished for approximately four weeks to maximise the proportion of small cell variants (SCV) to large cell variants (LCV) (Coleman, Fischer et al. 2004) as the more physically resistant variant is the one most likely to be found in the environment. Prior to purification the residual monolayer was scraped from the flask surface and homogenised in the culture medium. The cell suspension was then transferred to a 40 ml centrifuge tube which was centrifuged for 10 minutes at 40,000 × *g*.

Two methods were employed to purify the coxiellae from host cells. In the first method 5 ml of cell suspension in PBS was layered over 35 ml of a mixture of 30% sucrose and Urografin<sup>®</sup> 76% (Schering, NSW, Australia) with PBS as diluent and centrifuged at 18,000 × *g* for 30 minutes. The supernatant was removed by aspiration and the pellet resuspended in 5 ml phosphate buffered saline with 0.25 M sucrose (PBSS), pH 7.4 (Williams, Peacock et al. 1981).

In the second method cells were resuspended in sterile MilliQ water to induce osmotic shock and then passed through a 25 gauge needle 10 times to free coxiellae from host cell components and lyse any remaining LCV's. *Coxiella*

*burnetii* cells were purified from host cell debris using differential centrifugation (Baca, Aragon et al. 1981) which consisted of three alternating 10 minute cycles of low speed (820 x g) centrifugation followed by high speed (20,800 x g) centrifugation. Following the low speed centrifugation the pellet was discarded and the supernatant immediately subjected to the high speed step. Following the high speed step the supernatant was discarded and the pellet was resuspended in 500 µl of PBSS and then the cycle was repeated. The final cell pellet was resuspended in 500 µl PBSS.

### **2.2.9 Using optical density to estimate *Coxiella burnetii* genome number**

The absorbance of 32 individual cell suspensions, prepared as described above, were measured on a Nanodrop spectrophotometer at three wavelengths; 250<sub>nm</sub>, 420<sub>nm</sub> and 600<sub>nm</sub>. Genomic DNA was extracted from equal amounts of each sample using the modified Qiagen Tissue Mini-Kit procedure described previously. The resulting DNA samples were used as template for quantitative PCR using the IS1111a TaqMan assay. All samples were tested in duplicate. Calculation of correlation coefficients and drawing of scatter plots were done with Microsoft Excel XP Professional Edition. Results were expressed as genomes per micro litre of DNA eluate.

### **2.2.10 DNA extraction from environmental matrices**

#### **2.2.10.1 Extraction of DNA from bovine faeces and qPCR amplification**

Phase II *C. burnetii* was purified from infected tissue culture and purified as described previously. Cells suspended in PBS plus 0.25 M sucrose were stored at 4°C until required. Three 10-fold dilutions of the *C. burnetii* cell suspension were made just prior to use in PBS plus sucrose: 1 in 10, 1 in 100 and 1 in 1,000.



Approximately 25 grams of faeces was collected from four cows housed on the Murdoch University veterinary training farm that were shown to be serologically negative for *C. burnetii* infection using the CHEKiT Q fever ELISA (see Chapter 6 for details). One gram of each faecal sample was pooled and aliquots were made by dispensing 0.2 g of pooled faeces into each of 15 bead-beating tubes from a MO BIO UltraClean™ soil DNA isolation kit (MO BIO, Calsbad, California, USA) with the beads and bead solution removed and reserved in separate sterile tubes. A further 12 bead-beating tubes without faeces were prepared as above. Twenty microliters of each *C. burnetii* suspension was added to three separate tubes containing faeces and three containing no faeces. Additionally, 20 µl of PBS plus sucrose was added to three tubes containing faeces. Tubes containing faeces were mixed thoroughly by hand using a 200 µl pipette tip. Beads and bead solution were returned to all tubes and the DNA extraction was performed as per the manufacturers' instructions.

Five micro litres of DNA from each tube was amplified on a Rotorgene® 3000 machine using the TaqMan IS1111a assay described previously except with the addition of 10 µg of bovine serum albumin (Fisher Biotec Australia) to reduce inhibition by co-purified factors such as humic acid and tannins (Kreader 1996; Abu Al-Soud and Radstrom 2000). Microsoft Excel XP Professional Edition was used to manipulate and graph the data.

#### **2.2.10.2 Optimising DNA extraction conditions**

A series of experiments were performed to determine if the efficiency of the extraction of DNA from faeces could be improved by using the MO BIO PowerSoil™ DNA isolation kit. A pooled bovine faecal sample was made as described previously and 0.2 g aliquots were dispensed into 27 out of 36 bead

beating tubes with the beads and bead solution removed. Purified *C. burnetii* cells were prepared as described previously but were not serially diluted. Twenty micro litres of the *C. burnetii* cell suspension was dispensed into each of the bead beating tubes, mixed thoroughly with a 200µl pipette tip the beads and bead solution were returned to them. Solution 'C1' from the DNA isolation kit was added and all tubes were mixed on a MO BIO vortex genie with 2 ml tube adaptor head for 30 seconds.

The nine tubes containing *C. burnetii* cells without faeces were incubated at room temperature. The remaining 27 tubes containing faeces and purified *C. burnetii* cells were treated in one of four ways prior to continuing with the standard DNA extraction protocol. Six samples were placed in a heating block set to 70°C; nine samples were placed in a boiling water bath; nine were left at room temperature and three were subjected to three freeze/boil cycles with two minutes in liquid nitrogen and two minutes in boiling water. With the exception of the freeze/boil samples all tubes, including those without faeces, were incubated for five minutes at the designated temperature, mixed on the vortex genie for one minute and then incubated for a further five minutes at the same temperature as the first incubation. The standard DNA isolation procedure, as described in the product insert, was then followed from step five for all tubes. An analysis of variance (ANOVA) with Tukey's Honestly Significant Differences test at a 95% confidence limit was performed to determine which of the methods yielded the most *C. burnetii* DNA using the Statistical Package for Social Sciences (SPSS v. 15.0, SPSS Inc., Chicago, USA).

DNA from each tube was amplified on a Rotorgene<sup>®</sup> 3000 machine using the TaqMan IS1111a assay described previously with the addition of 10 µg of bovine serum albumin. Genomes per reaction, as estimated by the Rotorgene<sup>®</sup> 3000

software were expressed as a percentage of the control samples using Microsoft Excel and the means and standard errors of the means were then calculated using SPSS.

#### **2.2.10.3 Extraction of whole genomic DNA from kangaroo faeces and qPCR amplification**

The DNA was extracted from kangaroo faeces using the MO BIO PowerSoil™ DNA isolation kit and with a minor modification. Kangaroo faeces were collected from animals in the South West of Western Australia (Capel) as part of a commercial harvesting program. Details of the collection location can be seen in Chapter 6. Briefly, 0.2 g of faeces in bead-beating tubes were placed in a boiling water bath for five minutes, mixed on the Vortex Genie for one minute and then boiled for a further five minutes. The standard extraction protocol was then followed from step five onwards.

Two micro litres of each DNA sample was amplified on a Rotorgene® 3000 using the TaqMan IS1111a assay described previously. Genomes per reaction, as estimated by the Rotorgene® 3000 software were expressed as a percentage of the control samples using Microsoft Excel XP Professional Edition and the means and standard errors of the means were then calculated using SPSS. Results were graphed using Microsoft Excel.

#### **2.2.10.4 Extraction of whole genomic DNA from soil and qPCR amplification**

The procedure described for extraction of DNA from spiked kangaroo faecal samples using the MO BIO PowerSoil™ DNA isolation kit was repeated for soil samples collected from a cattle holding yard on the Murdoch University farm.

Five micro litres of each of the DNA extractions was amplified on a Rotorgene® 3000 machine using the TaqMan IS1111a assay described previously and data were analysed as described previously.

#### **2.2.10.5 Reduction of PCR inhibition**

The set of DNA samples extracted from bovine faecal samples, with *C. burnetii* cells added, and controls were amplified according to the standard conditions described previously except without the addition of 10 µg of bovine serum albumin to each reaction. This experiment was performed to confirm that addition of BSA improved the amplification efficiency of DNA in the system described.

Dilution of template was also performed to alleviate the effect of inhibitors co-extracted from the DNA samples. This was examined by two methods. First, 10 µl of each of the three bovine faecal samples which had the highest concentration of *C. burnetii* (1:10) added to them were pooled and diluted in two-fold steps to 1 in 64. Five micro litres of each was then subjected to qPCR amplification according to the conditions described previously except with the addition of 10 µg of bovine serum albumin to each reaction. Microsoft Excel XP Professional Edition was used to calculate a correlation coefficient between sample dilution and estimated *C. burnetii* genomes and from this a trend line was added to the scatter generated by the same program. The SPSS statistical package was used to conduct an analysis of variance with Tukey's Test for Honestly Significant Differences at a 95% confidence limit to determine if any level of template dilution was superior to the others.

The last method used to investigate the possible cause of the inhibition that was present in the samples was to extract DNA again from one of each of the

dilutions, including controls from the original experiment using the MO BIO PowerSoil™ DNA Extraction Kit. The exact volume of each sample was recorded before DNA extraction and this number was subsequently used to normalise qPCR results. Standard qPCR conditions were used except with the addition of 10 µg of bovine serum albumin to each reaction.

Pearson's correlation coefficient was calculated to determine the strength of the association between the sample dilution and estimated *C. burnetii* genomes. The significance of any differences in the estimated genomes in reactions containing different levels of template dilution were determined using an one-way ANOVA as described above.

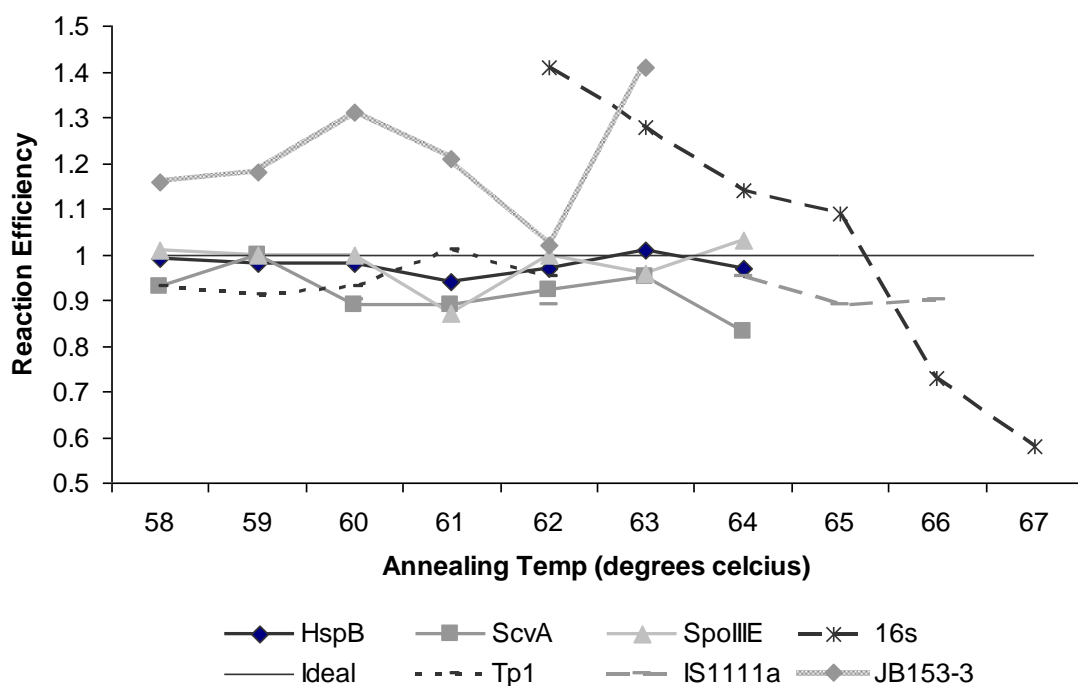
The last method used to investigate the possible cause of the inhibition that was present in the samples was to extract DNA again from one of each of the dilutions, including controls from the original experiment using the MO BIO PowerSoil™ DNA Extraction Kit. The exact volume of each sample was recorded before DNA extraction and this number was subsequently used to normalise qPCR results. Standard qPCR conditions were used except with the addition of 10 µg of bovine serum albumin to each reaction. The mean estimated genome number per reaction for each sample was expressed as a percentage of the positive control sample and an independent sample t-test with equal variances assumed was used to test the significance of any differences between primary and re-extracted samples at a given dilution.

## 2.3 Results

### 2.3.1 Optimising PCR Conditions

The reaction efficiencies of all assays with increasing annealing temperatures are shown in Figure 2.1 and the 'ideal' 1.0 efficiency is also plotted for comparison. Summaries of the optimum reaction conditions for each qPCR assay are presented in tables 2.3 to 2.5.

**Figure 2-1. Reaction efficiencies of seven qPCR assays at different annealing temperatures**



**Table 2-3. Optimum primer concentrations and annealing temperatures for qPCR assays**

<b>Assay</b>	<b>Forward Conc.</b>	<b>Reverse Conc.</b>	<b>Anneal Temp</b>
<b>IS1111a</b>	25 pmol	20 pmol	64°C
<b>TP1</b>	10 pmol	25 pmol	61°C
<b>FAF/RAF</b>	20 pmol	25 pmol	60°C
<b>HspB</b>	5 pmol	15 pmol	60°C
<b>SpoIIIE</b>	10 pmol	15 pmol	60°C
<b>JB153-3</b>	15 pmol	20 pmol	60°C
<b>ScvA</b>	20 pmol	15 pmol	59°C
<b>16S</b>	10 pmol	15 pmol	65°C

**Table 2-4. Optimum magnesium chloride and dual labelled probe concentrations for TaqMan qPCR assays**

<b>Assay</b>	<b>Mg<sup>2+</sup> Conc.</b>	<b>Probe Conc.</b>
<b>IS1111a</b>	4.5 mM	1.25 pmol
<b>TP1</b>	3 mM	5.00 pmol
<b>HspB</b>	3 mM	3.75 pmol
<b>SpoIIIE</b>	3 mM	1.25 pmol
<b>JB153-3</b>	3 mM	3.75 pmol
<b>ScvA</b>	3 mM	3.75 pmol
<b>16S</b>	3 mM	1.25 pmol



**Table 2-5. Optimum SYBR<sup>®</sup> Green and magnesium chloride concentrations for intercalating dye qPCR**

<b>Assay</b>	<b>Mg<sup>2+</sup> Conc.</b>	<b>SYBR Conc.</b>
<b>IS1111a</b>	5.5 mM	1 in 50,000
<b>TP1</b>	3.5 mM	1 in 50,000
<b>FAF/RAF</b>	5.0 mM	1 in 100,000

### **2.3.2 Reproducibility**

The standard error of the mean Ct values from the reproducibility experiments are summarised in tables 2.6 and 2.7. Overall lower errors were observed in the results from the TaqMan assays compared to the SYBR<sup>®</sup> Green assays. Using a bulk master mix marginally improved the reproducibility of one SYBR<sup>®</sup> Green assay. There was a significant decrease in the SEM of Ct values when a commercial TaqMan master mix was used.

**Table 2-6. Cycle threshold and SEM values for samples with known *Coxiella burnetii* DNA concentrations using TaqMan qPCR assays**

Genomes/ $\mu$ l	IS1111a		TP1		IS1111a MM	
	Ct	SEM	Ct	SEM	Ct	SEM
<b>5.56</b>	24.42	0.44	32.68	0.66	24.51	0.38
<b>5.56 x 10<sup>1</sup></b>	24.85	0.16	30.73	0.38	22.07	0.14
<b>5.56 x 10<sup>2</sup></b>	21.54	0.28	27.23	0.26	19.17	0.07
<b>5.56 x 10<sup>3</sup></b>	18.37	0.17	23.20	0.21	15.73	0.05
<b>5.56 x 10<sup>4</sup></b>	15.55	0.22	20.59	0.18	12.29	0.05
<b>5.56 x 10<sup>5</sup></b>	12.25	0.12	16.99	0.19	8.70	0.06
<b>5.56 x 10<sup>6</sup></b>	8.42	0.14	13.41	0.19	5.38	0.06

**Table 2-7. Cycle threshold and SEM values for samples with known *Coxiella burnetii* DNA concentrations using SYBR<sup>®</sup> Green I qPCR assays**

Genomes/ $\mu$ l	IS1111a		TP1		FAF/RAF		FAF/RAF MM	
	Ct	SEM	Ct	SEM	Ct	SEM	Ct	SEM
<b>5.56</b>	25.04	1.26	29.54	1.01	32.83	0.74	33.27	0.99
<b>5.56 x 10<sup>1</sup></b>	25.37	0.82	28.35	0.46	31.06	0.52	32.57	0.54
<b>5.56 x 10<sup>2</sup></b>	21.19	1.12	25.14	1.05	26.46	0.38	27.27	0.28
<b>5.56 x 10<sup>3</sup></b>	18.27	1.44	21.22	1.00	22.74	0.44	24.84	0.12
<b>5.56 x 10<sup>4</sup></b>	15.73	0.87	20.01	0.73	21.17	0.28	22.73	0.12
<b>5.56 x 10<sup>5</sup></b>	10.32	0.53	15.35	1.08	16.79	0.32	19	0.17
<b>5.56 x 10<sup>6</sup></b>	7.03	0.25	11.66	0.56	13.48	0.35	15.51	0.27

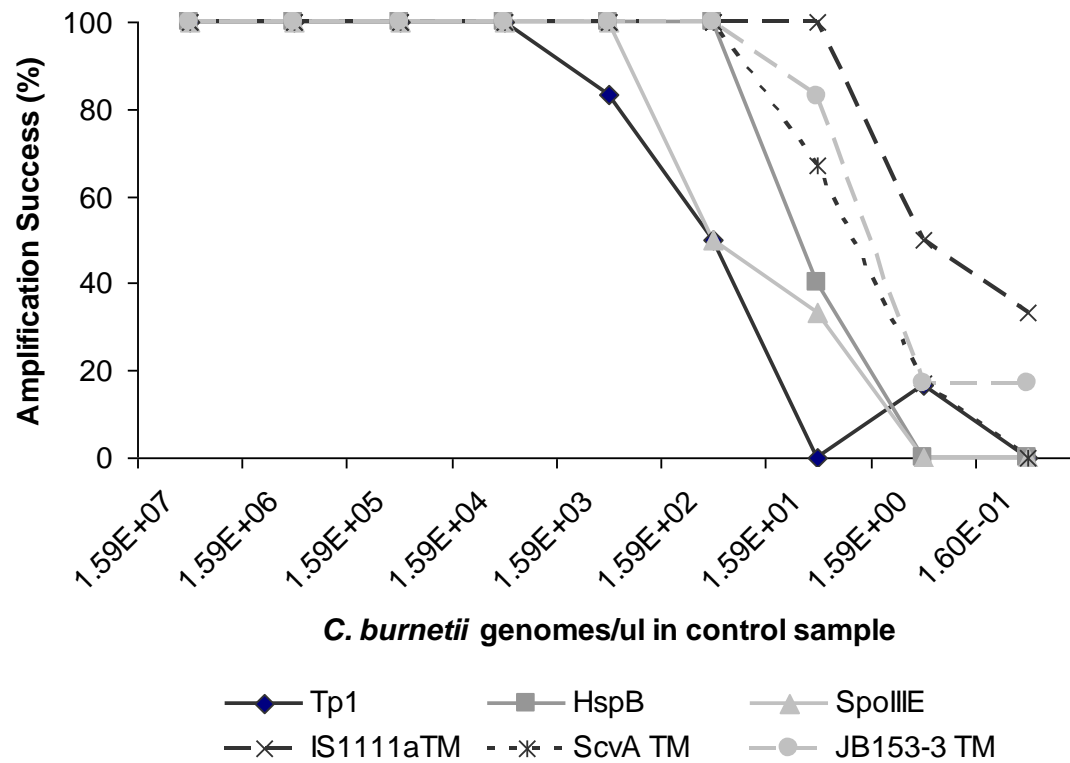
### 2.3.3 Analytical sensitivity

The analytical sensitivity of all TaqMan assays was determined by analysing several factors; (1) the coefficient of variation of estimated genome equivalents for each dilution of DNA (Table 2.8); (2) the DNA dilution at which greater than, or equal to, 50% of the reactions failed (Figure 2.2) and; (3) the linearity of the Ct values for each assay (Figure 2.3). Based on these criteria the IS1111a assay was the most sensitive with a theoretical detection limit of 0.16 genomes per micro litre of template added. However, detection of this low concentration of DNA was not repeatable and did not give a reliable estimate of DNA concentration because the relationship between the cycle threshold value and the given *C. burnetii* DNA concentration was not linear. The lowest DNA concentration that could be reliably detected (that is; produce detectable amplification greater than 50% of the time) with the IS1111a assay was 15.9 genomes per micro litre. The JB153-3 test was also able to detect as few as 0.16 genomes per reaction but was less reliable and had inferior coefficients of variation in comparison to the IS1111a assay.

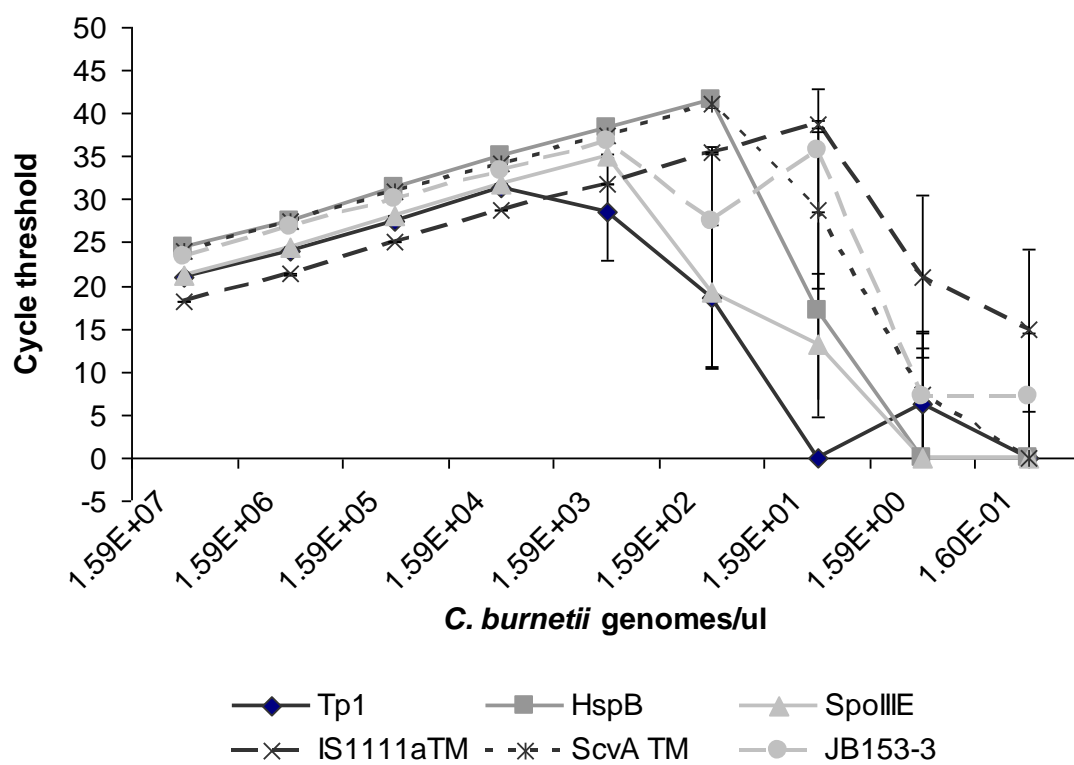
**Table 2-8. Coefficients of variation (%) of estimated *Coxiella burnetii* genome equivalents resulting from qPCR amplification of a set of DNA standards diluted in 10-fold steps**

<b>Genomes/<math>\mu</math>l</b>	<b>Tp1</b>	<b>HspB</b>	<b>SpoIIIE</b>	<b>IS1111a</b>	<b>ScvA TM</b>	<b>JB153-3</b>
<b>1.59E+07</b>	12.56	10.03	6.86	1.84	18.73	18.16
<b>1.59E+06</b>	9.85	6.85	3.57	2.59	21.97	13.37
<b>1.59E+05</b>	5.13	18.31	4.00	2.08	13.35	9.39
<b>1.59E+04</b>	3.51	8.03	7.96	3.64	11.79	14.86
<b>1.59E+03</b>	60.66	26.70	29.08	6.70	24.76	26.60
<b>1.59E+02</b>	171.85	82.84	122.25	6.22	64.87	111.99
<b>1.59E+01</b>	-	137.29	192.05	77.39	91.38	59.44
<b>1.59E+00</b>	244.95	-	-	153.35	244.95	244.95
<b>1.60E-01</b>	-	-	-	239.50	-	244.95

**Figure 2-2. Percentage of reactions that showed detectable amplification of 10-fold dilutions of *Coxiella burnetii* DNA with different qPCR assays**



**Figure 2-3. Cycle threshold values versus *Coxiella burnetii* DNA standard concentration for six qPCR assays**





#### **2.3.4 Analytical specificity**

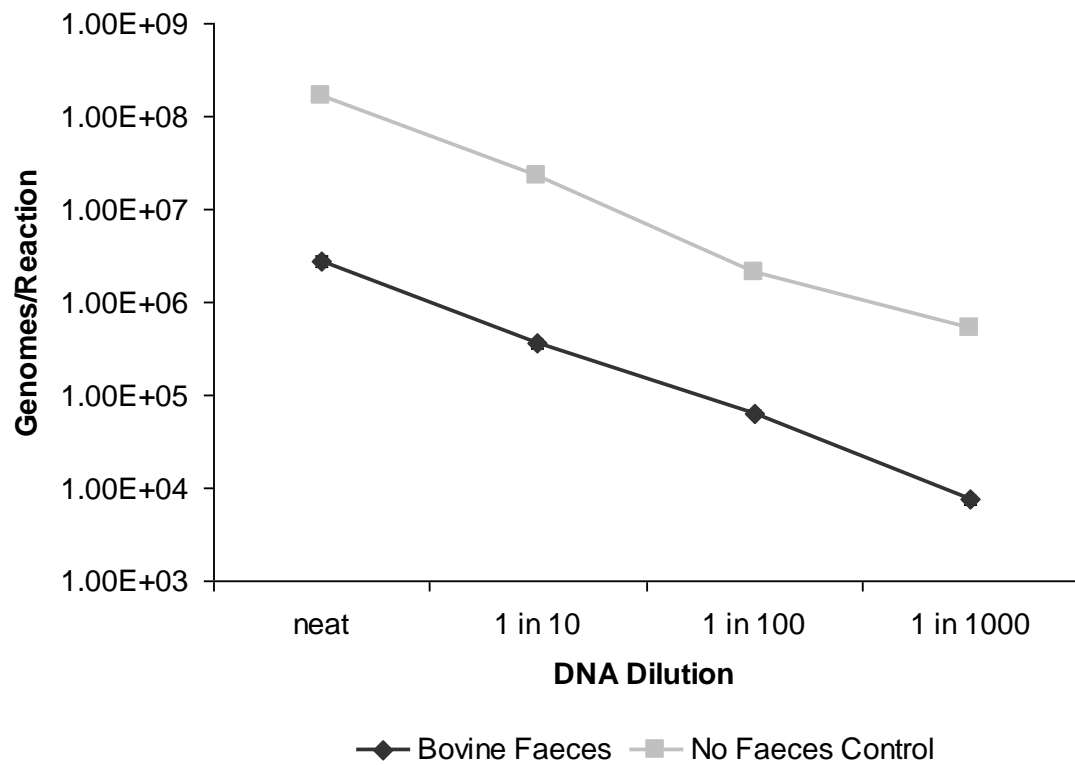
None of the DNA samples tested produced detectable amplification with the IS1111a, JB153-3, Tp1, HspB, ScvA or SpolIIE assays by cycle 40. The 16S assay produced detectable amplification for DNA samples from all organisms except *S. aureus*, *S. equii* and *R. rickettsii*.

#### **2.3.5 Extraction of *Coxiella burnetii* DNA from environmental matrices**

##### **2.3.5.1 Extraction of DNA from bovine faeces and qPCR amplification of *Coxiella burnetii* DNA**

The qPCR results generated from this experiment indicate that there was over 60 times as much amplifiable DNA present in the controls as in the corresponding spiked bovine faecal samples. The relationship between *C. burnetii* genomes per micro litre and the dilution of a *C. burnetii* cell suspension is shown in Figure 2.4.

**Figure 2-4. Graph showing the reduced qPCR-estimated *Coxiella burnetii* DNA concentration in faecal samples in comparison to the DNA concentration estimated for buffer controls**

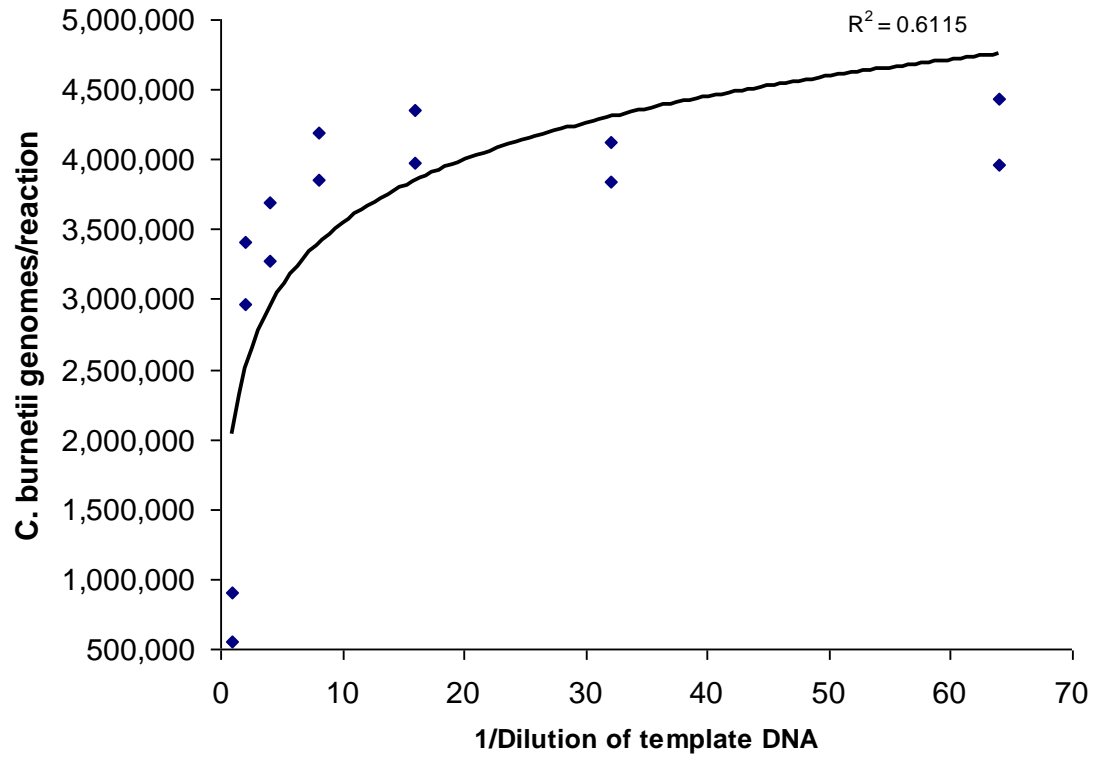


### 2.3.5.2 Reduction of PCR inhibition

The addition of 10 µg of bovine serum albumin improved the efficiency of the PCR reaction from approximately 60% success to 100% success.

Adjustment of the raw data showed that there was a direct correlation between the increasing dilution and decreasing inhibition as evidenced by an increase in the estimated *C. burnetii* DNA concentration in more dilute samples. The  $R^2$  value for the correlation between the previously mentioned factors was 0.6115. A graph showing the relationship between DNA dilution and inhibition of PCR is shown in Figure 2.5. The greatest relief of inhibition was obtained by diluting the template one to one with water. Accurate correction for the dilution factor of each sample was only possible when the estimated genomes for a particular sample fall within the linear range of the standard curve used. All PCR results for the samples in this experiment fell within the linear range of the standard curve.

**Figure 2-5. Correlation between the dilution of template and estimated *Coxiella burnetii* genomes per reaction as estimated by qPCR**



A maximum reduction in inhibition was seen with template that had been diluted 1 in 64 before being subjected to amplification. Similar levels of relief of inhibition were observed when template was diluted 1:8, 1:16 and 1:32. The greatest change in relative inhibition occurred between the neat and 1:2 samples where, after adjustment for the dilution factor, there was estimated to be nearly 4.5 times as much *C. burnetii* DNA in the diluted sample than in the neat samples. All qPCR estimates of *C. burnetii* DNA concentration from diluted template were significantly greater than for the undiluted sample but there were no significant differences between diluted samples ( $p < 0.001$ ).

For samples that were subjected to a second round of DNA purification using the PowerSoil™ DNA isolation kit there was significantly more *C. burnetii* DNA detected in the samples purified initially compared to the samples after a second purification was performed at a dilution of 1 in 100 ( $p < 0.05$ ). No other differences were observed.

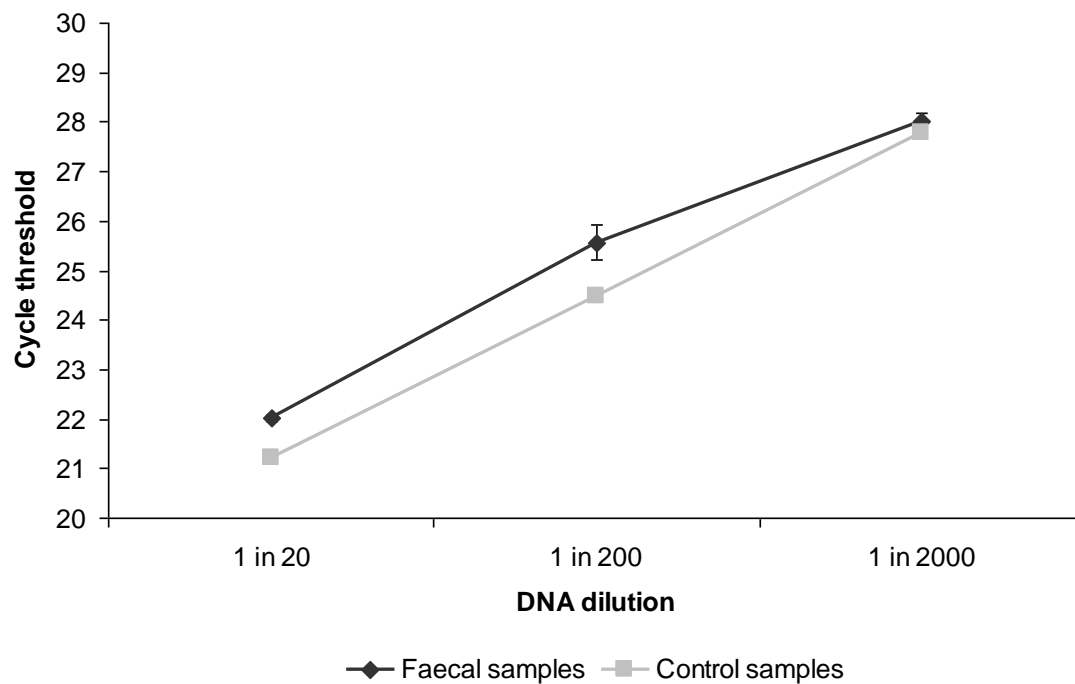
#### **2.3.5.3 Optimising DNA purification conditions**

There was no significant difference in the DNA yield when samples were purified using the standard procedure with and without the addition of a 70°C incubation. There was significantly more DNA purified when samples were boiled or freeze-thawed prior to purification ( $p < 0.001$ ). There was no significant difference in the DNA yield obtained using either boiling or freeze-thawing before purification. Due to its simplicity and the good recovery noted above the boiling method was used for further experiments.

#### **2.3.5.4 Efficiency of purification of whole genomic DNA from kangaroo faeces and qPCR amplification**

The Ct values from the qPCR results from this experiment are displayed in Figure 2.6. Overall, when using the modified DNA purification method described previously it was possible to purify then amplify greater than 70% of *C. burnetii* DNA from kangaroo faeces compared to DNA purification from buffer alone.

**Figure 2-6. Comparison of cycle threshold values for a quantitative PCR used to detect *Coxiella burnetii* DNA extracted from kangaroo faeces and from buffer**

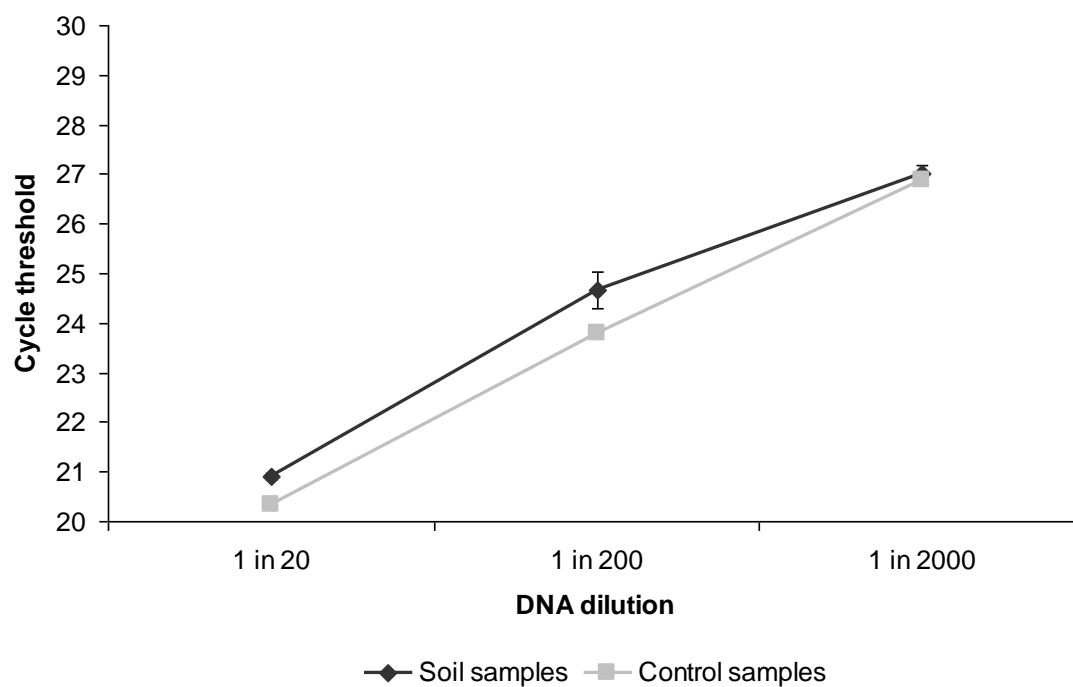


#### **2.3.5.5 Purification of whole genomic DNA from soil and qPCR amplification**

The Ct values from the qPCR results from this experiment are displayed in figure 2.7. Overall, when using the modified DNA purification method described previously it was possible to extract and amplify greater than 72% of *C. burnetii* DNA from kangaroo faeces in comparison to DNA purification from buffer alone.



**Figure 2-7. The differences between qPCR cycle threshold values for *Coxiella burnetii* DNA extracted from soil and PBS**



### 2.3.6 Purification of *Coxiella burnetii* from vero cell monolayers

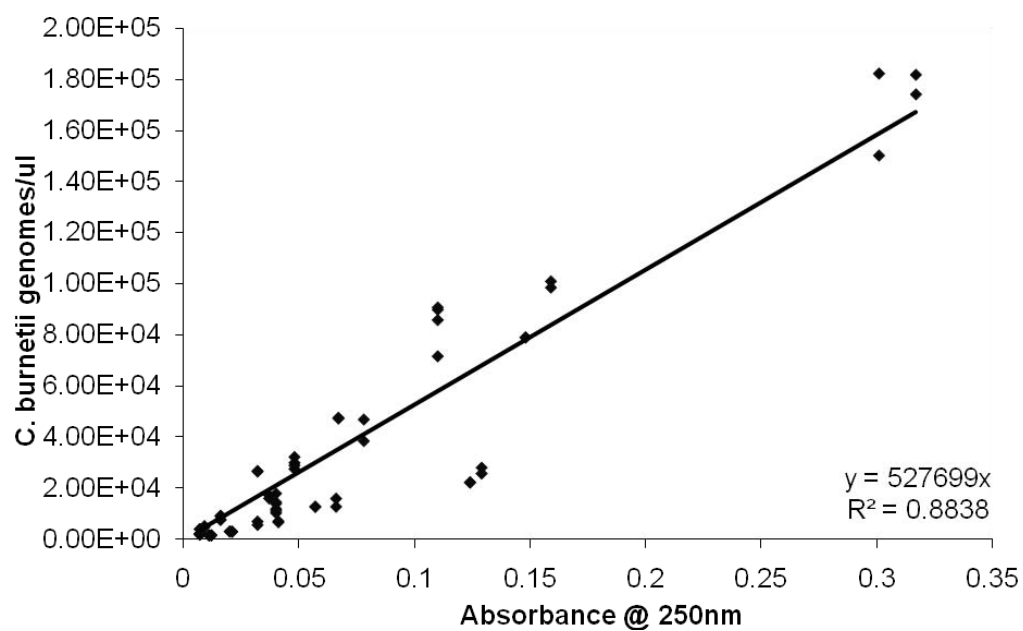
The method of Williams et al. (1981) provided inconsistent recovery of *C. burnetii* from infected vero cell monolayers. Residual Urografin in the cell preparation also interfered with the measurement of the absorbance of the cell suspension required to quantifying *C. burnetii*.

The differential centrifugation method was repeatable and resulted in a cell suspension that appeared to be free of host cell components. This method was used to purify *C. burnetii* for the subsequent experiments and used to correlate the absorbance of the cell suspensions with genome equivalents estimated with qPCR.

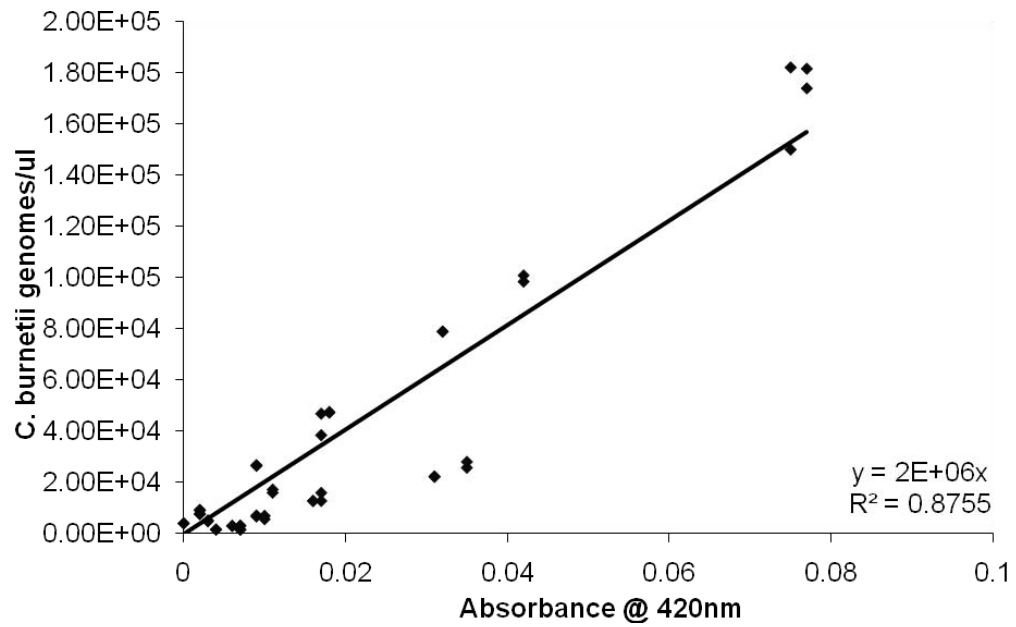
### 2.3.7 Regression analysis of genome copy number versus Abs<sub>250nm</sub>

There was a strong correlation ( $R^2=0.89$ ) between the absorbance at a wavelength of 250 nm and estimated genome number obtained from testing DNA purified from *C. burnetii* with a qPCR (Figure 2.8). Strong correlations were also observed between estimated genome number and absorbance at 420 nm ( $R^2=0.88$ ) and 600 nm ( $R^2=0.65$ ), which are shown in figures 2.9 and 2.10 respectively.

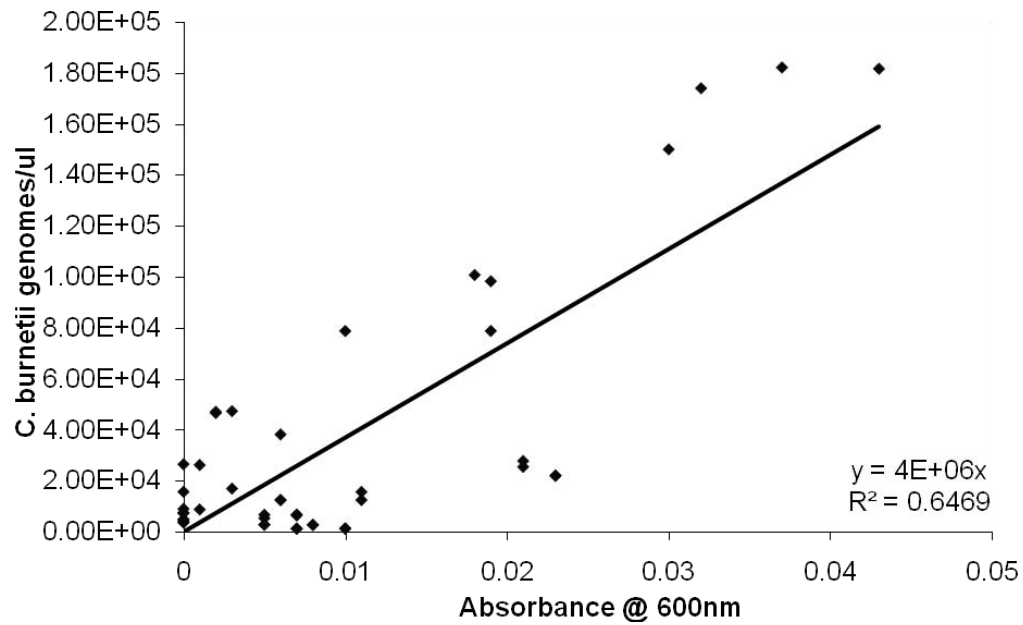
**Figure 2-8. The correlation between the absorbance of a suspension of *Coxiella burnetii* cells at 250nm and the genome number estimated by qPCR**



**Figure 2-9. The correlation between the absorbance of a suspension of *Coxiella burnetii* cells at 420nm and the genome number estimated by qPCR**



**Figure 2-10. The correlation between the absorbance of a suspension of *Coxiella burnetii* cells at 600nm and the genome number estimated by qPCR**



## 2.4 Discussion

Two different detection chemistries were compared in this study: the non-sequence specific SYBR<sup>®</sup> Green I detection and the sequence specific TaqMan detection system. The results of this study are in agreement with other studies that showed that a qPCR using sequence specific fluorophore-labelled oligonucleotide probes has greater specificity and reliability compared to a qPCR using intercalating dye-based methods (Bustin and Nolan 2004). However, other DNA intercalating dyes such as SYTO9 appear to have improved reproducibility in comparison to SYBR Green I (Monis, Giglio et al. 2005) and these dyes may have produced a qPCR that compared better to the TaqMan qPCR.

Optimising magnesium chloride concentrations was more critical and variable for SYBR<sup>®</sup> Green-based assays compared to the TaqMan assays. A reaction concentration of 3 mM MgCl<sub>2</sub> was optimal for all TaqMan assays except the IS1111a primer/probe set which required 4.5 mM. It was also found that titration of annealing temperatures had a noticeable effect on both the specificity and efficiency of the reactions. Lower annealing temperatures in some cases resulted in no template control reactions producing detectable fluorescence while higher temperatures were deleterious to reaction efficiency as evidenced by generally poor efficiencies observed for temperatures at or above 63°C. Therefore, it seems that accurate titration of annealing temperature has the largest impact on the sensitivity and specificity of these quantitative PCR's.

A range of gene targets have been used in different PCR assays to detect *C. burnetii*. These targets include the outer membrane protein gene *com1* (Brennan and Samuel 2003), the superoxide dismutase gene (Stein 1992; Masala, Porcu et al. 2004) and the *IS1111a* insertion sequence (Berri, Laroucau et al. 2000; Klee,

Tyczka et al. 2006). There has been some debate over the usefulness of the *IS1111a* for detection of *C. burnetii* (Marmion, Storm et al. 2005; Rolain and Raoult 2005). However, it makes an attractive target due to its high copy number and lack of significant homology to published sequences from other related organisms (Hoover, Vodkin et al. 1992). The variability exhibited by this element between strains of *C. burnetii* with regards to copy number has been exploited for a genotyping method (Denison, Thompson et al. 2007), which suggests that a qPCR targeting this element may not be quantitative for unknown isolates. There have also been conflicting reports regarding whether or not *Coxiella*-like organisms possess the gene encoding the 27 kD outer membrane protein (Lee, Park et al. 2004). However, published data seem to suggest that the *IS1111a* transposon is specific for *C. burnetii* species (Hoover, Vodkin et al. 1992; Reeves, Loftis et al. 2005).

The results from comparing different primer sets showed that the qPCR targeting the *IS1111a* transposon was the most sensitive and reproducible. The reproducibility of this test was further enhanced through the use of a commercial TaqMan master mix (Invitrogen Platinum<sup>®</sup> Quantitative PCR SuperMix-UDG). This master mix also prevents amplification of carried over PCR products by incorporating dUTP during strand synthesis and employing a pre-PCR digestion of dUTP-containing sequences (Kwok and Higuchi 1989). The *IS1111a* assay was able to detect 15.9 genomes per micro litre of buffer in 100% of samples. The variability of the qPCR increased at DNA concentrations lower than 15.9 genomes/ $\mu$ l. In absolute terms the assay was able to detect 0.16 genomes/ $\mu$ l in one third of the samples tested. This extremely low level of DNA would only be detected confidently if the number of replicates tested was increased or the volume of template used was increased. It is unlikely that this assay would still be quantitative when detecting such a low concentration of DNA.

During this study a qPCR utilising TaqMan detection chemistry was designed to ensure that any PCR positives detected did not result from contamination by the *C. burnetii* phase II strain RSA439 (clone 4) that was cultured in a nearby laboratory for other experiments. This PCR targeted an ORF putatively named JB153-3 which is present in all eight phase I *C. burnetii* isolates for which significant sequence data are available but is absent from clone 4, NM phase II clone 1, NM Baca and RSA 514 due to a chromosomal deletion (Hoover, Culp et al. 2002; Denison, Massung et al. 2007). This putative ORF has very little similarity to other sequences currently found in the NCBI GenBank database. This was the only other qPCR that was sensitive enough to successfully amplify 0.16 genomes in one of six samples. Laboratories that perform diagnostic PCR for the detection of *C. burnetii* and concurrently culture phase II coxiellae for other experiments need to be extremely careful that contamination of test samples does not occur. The JB153-3 qPCR described here could be a valuable tool for ensuring the validity of test results because of the highly persistent nature of *C. burnetii* (PHAC 2001) and the ease with which it can be disseminated in a laboratory environment (Babudieri 1959). However, it must be noted that this PCR targets a redundant sequence in the *C. burnetii* genome (Hoover, Culp et al. 2002) and this sequence may not be conserved in all wild-type strains. To date some phase II and intermediate phase strains have been shown to retain this genomic region (Denison, Massung et al. 2007). In addition, *C. burnetii* DNA extracted from Q-Vax vaccine was used to make standard curves for the qPCR assays described here to ensure that only small quantities of template DNA were present in the laboratory. This measure was taken to reduce the potential for contamination of samples, which can result from the high concentrations of DNA that are generated when producing cloned sequences for standard curve construction (Marmion, Storm et al. 2005).



Other than the 16S qPCR, none of the assays tested amplified DNA from any of the specificity controls used after a maximum of 40 PCR. Beyond 40 cycles non-specific amplification sometimes occurred, particularly with SYBR<sup>®</sup> Green I detection, but this was not investigated in any detail. The 16S assay produced detectable amplification for DNA samples from all organisms except *S. aureus*, *S. equii* and *R. rickettsii*.

The optimised IS1111a assay was used to evaluate the effectiveness of two MO BIO DNA extraction kits; the UltraClean<sup>™</sup> kit and the PowerSoil<sup>™</sup> kit. DNA isolation kits produced by MO BIO have been found to be effective in isolating DNA from a variety of environmental substrates (Klerks, van Bruggen et al. 2006; Whitehouse and Hottel 2007). However, all assessments have been based on data produced when using the standard procedures provided by the manufacturers. In this study it was found that the standard procedure recommended by the manufacturer was insufficient for purification of DNA from a faecal substrate. Klerks et al. (2006) postulated that the aggregation of *Salmonella enterica* on or between substrate particles could make efficient DNA extraction from soil or faecal samples difficult. A similar problem could exist with *C. burnetii* in soil and faeces and could have been exacerbated due to the use of phase II cells which have a tendency to clump together (Williams, Peacock et al. 1981; Coleman, Fischer et al. 2007). The addition of a boiling step at the beginning of the procedure significantly improved the efficiency of DNA purification. The effect of co-purified inhibitors on the qPCR was also assessed and alleviated through the addition of bovine serum albumin and dilution of template.

The differential centrifugation method used in this study (Baca, Aragon et al. 1981) provided reliable recovery of *C. burnetii* from vero cell monolayers.

However, the overall efficiency of the method was not investigated because it was outside the scope of the current study. Experiments could be conducted to measure the amount of *C. burnetii* DNA present in each discarded fraction relative to the retained fraction to help quantify the recovery. However, the results from this study suggest that this is a simple and relatively rapid way to purify *C. burnetii* from host cell components.

It is difficult to perform quantitative *in vivo* and *in vitro* experiments using *C. burnetii* because it is small in size and an obligate intracellular pathogen. The high correlation of the estimated genomes/ $\mu$ l with the absorbance at 250 nm and 420 nm shows that spectrophotometry is a useful, cheap and rapid method of enumerating *C. burnetii*. These results show that the optimum wavelength for estimating *Coxiella* cell density is 250 nm. Spectrophotometric methods of quantifying *C. burnetii* at both 420 (Kovacova, Kazar et al. 1998) and 600 nm (Zhang and Samuel 2003) have also been employed but the methods employed are not described in detail. It is possible that both groups based their methods upon an earlier study in which a correlation was made between the turbidity reading obtained with a Klett-Summerson photoelectric colorimeter at 420 nm (blue filter), PFU and direct counts (Williams, Peacock et al. 1981). However, without the specialist equipment listed previously it is not possible to repeat the methods referred to. Immuno-staining (Schneider 1989) and plaque assays (McDade and Gerone 1970) have also been used to estimate *Coxiellae* numbers but both assays are labour and time intensive. Furthermore, phase II *C. burnetii* cells are highly hydrophobic due to the truncated nature of the LPS produced by this phase variant (Williams, Peacock et al. 1981; Coleman, Fischer et al. 2007). This causes the cells to clump together and may confound enumeration by methods such as those mentioned previously.

## 2.5 Conclusions

This study describes the thorough optimisation of several quantitative PCR assays and provides evidence to support the selection of one of these assays as superior to the others. The qPCR targeting the *IS1111a* insertion sequence and utilising the TaqMan detection system was the most sensitive and reliable. A commercial master mix further enhanced the reproducibility of the test. The assay was compatible with DNA purified from both faeces and soil with little to no PCR inhibition. This study also developed a simple, rapid method for enumerating viable *C. burnetii* cells *in situ* based on spectrophotometric measurement of absorbance at 250 nm. The findings of this study are specific for the *C. burnetii* cell purification and DNA isolation techniques that were used but would be readily adaptable to other procedures.

### **3. A cell culture-based quantitative-PCR assay to determine the susceptibility of *Coxiella burnetii* to chemical and physical disinfectants**

#### **3.1 Introduction**

*Coxiella burnetii* has at least two morphologically distinct cellular forms: the metabolically active large cell variant (LCV) and the chemically resistant and environmentally stable small cell variant (SCV) (Wiebe, Burton et al. 1972; McCaul, Banerjee-Bhatnagar et al. 1991; Coleman, Fischer et al. 2004). The SCV is very resistant to physical and chemical insults including elevated temperature and pressure, desiccation, osmotic shock and several chemical disinfectants (Ransom and Huebner 1951; Malloch and Stoker 1952; McCaul, Hackstadt et al. 1981; McCaul, Banerjee-Bhatnagar et al. 1991; Cerf and Condron 2006). The physically resistant nature of *C. burnetii* means that it can persist in the environment for a long time. Therefore, environmental substrates such as soil, dust and water that have been contaminated by domestic livestock can pose a risk of infection to humans for many months after they were originally contaminated with *C. burnetii*.

There are no validated methods for inactivating *C. burnetii* in liquid wastes associated with domestic livestock. Therefore, it is important to evaluate disinfection agents and treatments that can be used to inactivate *C. burnetii* on a large scale in the waste produced by livestock-associated industries.

Animal bioassays are currently used for routine assessment of the viability and infectivity of *C. burnetii* (Arricau-Bouvery, Souriau et al. 2005) and other obligate intracellular organisms (Shin, Linden et al. 2001). However, animal assays are

expensive, laborious, give variable estimates of infectious dose and raise ethical questions about the use of animals when an alternative method could be developed (Ransom and Huebner 1951; Malloch and Stoker 1952; Sobsey and Leland 2001). Previous *C. burnetii* disinfection studies have used the immunological response of infected laboratory animals post-infection and serial passage through embryonated eggs to provide information on how effective an antimicrobial agent is (Ransom and Huebner 1951; Little, Kishimoto et al. 1980; Scott, McCaul et al. 1989; Scott and Williams 1990). These two methods are highly sensitive but they are very time consuming and provide only indirect quantitative information on how effective a particular agent is. The viability of *C. burnetii* can also be estimated using plaque assays (McDade and Gerone 1970; Wike, Tallent et al. 1972; Schneider 1989). However, while this type of assay has been found to give results comparable with direct counts and 50% infectious dose methods ( $IC_{50}$ ), their use with *C. burnetii* is problematic because plaques take a long time to develop and can be difficult to identify due to their irregular shape and small size (Wike, Tallent et al. 1972). Moreover, plaque assays are not able to be used with 'dirty' samples without substantial sample sterilisation beforehand which may confound results. The cell culture-based system described obviates the need to use laboratory animals or plaque assays and provides reliable quantitative data on the reduction in viability caused by an antimicrobial agent.

### **3.2 Materials and methods**

#### **3.2.1 Culture and extraction of *Coxiella burnetii* from tissue culture cells**

Vero (African green monkey kidney) cells infected with *C. burnetii* Phase II Nine Mile RSA439 (clone 4) (see section 2.2.8) were grown in 25 cm<sup>2</sup> culture flasks (TPP, Switzerland) at 37°C with 5% CO<sub>2</sub> (v/v) in DMEM (MP Biomedicals, Australia) supplemented with 200mM L-Glutamine (MP Biomedicals, Australia) and

2% v/v foetal calf serum. Cultures were grown without replenishment of media for approximately four weeks to maximise the proportion of SCV to LCV (Coleman, Fischer et al. 2004). Prior to purification the residual monolayer was scraped from the flask surface and homogenised in the cell culture medium. The cell suspension was then transferred to a centrifuge tube and centrifuged for 10 minutes at  $40,000 \times g$ . Cells were resuspended in sterile MilliQ water to induce osmotic shock and then passed through a 25 gauge needle 10 times to free *Coxiellae* from host cell components and lyse any remaining LCV's. *Coxiella* cells were purified from host cell debris using differential centrifugation (Baca, Aragon et al. 1981) which comprised three cycles of low speed ( $820 g$  for 10 minutes) centrifugation followed by high speed ( $20,800 \times g$  for 10 minutes) centrifugation. The final resuspension was made in sterile highly-pure (HP) water and the concentration of *C. burnetii* cell suspensions were adjusted to approximately  $23,000 \pm 2,000$  *C. burnetii* cells per microliter according to the method described in Chapter 2 and 50  $\mu$ l aliquots were dispensed into 1.5 ml microfuge tubes.

### 3.2.2 Treatment medium

Disinfectant treatments were initially assessed in highly pure (HP) sterile water. Treatments that appeared to be effective were subsequently evaluated in wastewater collected from a drainage sump on the Murdoch University farm, which collected runoff from fields and livestock holding yards. The water, hereafter referred to as 'wastewater', was strained through 100 micron mesh to remove large particulate matter and autoclaved. The resulting liquid was assessed for the following parameters: pH, turbidity, salinity/conductivity (S/C), total organic carbon (TOC) and total dissolved solids (TDS) by the Marine and Freshwater Research Laboratories (MAFRL, Murdoch University). Wastewater was kept at 4°C until required and then allowed to equilibrate to room temperature before being used.

The pH of the HP water and wastewater after addition of each chemical disinfectants was measured using a TPS WP-80 pH-mV-Temp. Meter (Springwood, Brisbane, Australia).

### **3.2.3 Treatment with disinfectants**

Each treatment and control group were assayed in triplicate. *Coxiella burnetii* cells that had not been exposed to the treatment (NT) and *C. burnetii* cells which had been completely inactivated (Kill) were included with every plate. No treatment controls were exposed to the same conditions as the treated samples except the agent was not included. Sterile HP water was added to the NT control in place of the chemical agent and NT controls were left in normal light conditions when UV treatment was used. Four treatments were applied to *C. burnetii* cells in various experiments to serve as complete inactivation (Kill) controls of: (1) incubation in 3 M NaOH at room temperature for 30 minutes; (2) incubation in 20 mM NaOCl at room temperature for 30 minutes; (3) exposure to 300 mJsec<sup>-1</sup> of ultra violet radiation at room temperature and (4) incubation at 95°C for 30 minutes. For kill control (1) the samples were washed in warm DMEM twice following exposure. For kill control (2) reactive oxygen species were neutralised with 20 µl of 7g/L sodium thiosulphate following exposure. The kill control treatment that was used for each experiment is shown in the results table corresponding to that treatment. All controls and treated samples were performed in triplicate.

#### **3.2.3.1 Experiment 1: sodium hypochlorite treatment**

Two separate experiments were performed to assess the efficacy of NaOCl in buffer at different concentrations with a constant exposure time. Both followed the same general protocol as follows: an equal volume of NaOCl pre-diluted in sterile

HP water was added to 50 µl of *C. burnetii* suspension in 1.5 ml microfuge tubes. Tubes were mixed and allowed to stand at room temperature for 30 minutes. Addition of 20 µl of 7g/L sodium thiosulphate was used to neutralise the reactive species at the end of the 30 minute exposure. For experiment 1.2 one set of samples was subjected to RNA extraction for assessment of viability using the RT-qPCR that is described in Chapter 3. Three hundred microliters of warm DMEM (with L-glutamine and 2% FCS) was then added to all remaining samples and each cell suspension was used to inoculate one well in a 24 well tissue culture plate containing confluent vero cells. The same procedure was used for a subsequent experiment with the *C. burnetii* cells suspended in wastewater prior to the addition of NaOCl.

The relationship between NaOCl efficacy and exposure time was also evaluated using the same procedure described previously as follows: cell suspensions were exposed to 0.05 mM NaOCl and 20 µl of 7g/L sodium thiosulphate was added 5, 10, 20 and 30 minutes after to the addition of NaOCl to quench oxidative species. The concentrations of NaOCl assessed in each experiment are shown in Table 3.2.

### **3.2.3.2 Experiment 2: ultra-violet (UV) light treatment**

Three experiments were used to assess the susceptibility of *C. burnetii* to ultra violet (UV) light. The dose of radiation delivered and the kill control treatments used in each experiment are presented in Tables 3.4 and 3.5 respectively. The same general procedure was followed for all experiments: *Coxiella burnetii* cell suspensions were dispensed into clear microcentrifuge tubes and exposed to UV light using a GeneLinker UV illumination chamber (BioRad, Regents Park, New South Wales, Australia). For experiment 2.3 RNA was purified from one set of



samples for assessment of viability using RT-qPCR as described in Chapter 3. Three hundred microliters of warm DMEM (with L-glutamine and 2% FCS) added to each remaining cell suspension and each suspension used to inoculate one well in a 24 well tissue culture plate containing confluent vero cells. To ensure that the UV dose delivered was consistent the GeneLinker UV tubes were pre-warmed by delivering a dose of 300 mJ/cm<sup>2</sup> before the test samples were exposed. Cultures were incubated overnight at 37°C with 5% CO<sub>2</sub> (v/v) to allow internalisation of viable *C. burnetii* cells. All wells were subsequently washed three times with warm culture medium to remove any dead *C. burnetii* cells. Plates were incubated for a further seven days.

### 3.2.3.3 Experiment 3: ozone treatment

A custom-made ozone generator was used to produce ozone for these experiments. The apparatus utilised a pair of electrodes to split O<sub>2</sub> molecules into oxygen radicals via corona discharge. The aqueous ozone concentration produced by the apparatus when 500 ml/min of ambient air was passed between the electrodes was approximately 12 mg/L determined using the indigo method (Bader and Hoigne 1981). When the same flow rate of compressed oxygen was passed between the electrodes the aqueous ozone concentration produced was 43 mg/L. *Coxiella burnetii* suspensions were made up to 30 ml in PBSS and dispensed into the exposure chamber. A mixture of air or compressed oxygen and ozone was then bubbled through the cell suspension. Nine hundred and ninety micro litre samples were withdrawn at the desired time points and added to 20 µl of 7 g/L sodium thiosulfate solution to quench residual ozone. Cells were pelleted by centrifugation, resuspended in warm cell culture medium and inoculated onto confluent monolayers of vero cells as described previously. No treatment controls

were subjected to the same conditions as described above except the ozone generator was not turned on. Cultures were incubated as described above.

#### **3.2.3.4 Experiment 4: Peracetic acid treatment**

Equal volumes of 32, 20 and 10 % v/v PAA diluted in sterile HP water were added to 50 µl of *C. burnetii* suspension in 1.5 ml microfuge tubes (final concentrations were 16, 10 and 5 % v/v respectively). Tubes were mixed and allowed to stand at room temperature for 60 minutes and subsequently washed, resuspended in 300 µl of warm DMEM (with L-glutamine and 2% FCS) added to them and each cell suspension was used to inoculate one well in a 24 well tissue culture plate containing confluent vero. Cultures were incubated as described above.

#### **3.2.3.5 Experiment 5: treatment with peracetic acid in combination with sodium hypochlorite**

The synergistic effect of treating *C. burnetii* cells with a combination of peracetic acid (PAA) and NaOCl was investigated in both sterile HP water and wastewater. *Coxiella burnetii* cells were purified as described previously and added to equal volumes of  $6.4 \times 10^{-5}\%$  PAA in 1.5 mL micro-centrifuge tubes, mixed well and incubated at room temperature for 60 minutes. An equal volume of pre-diluted NaOCl was then added to each group of tubes to achieve the desired hypochlorite concentration. All dilutions were made in either sterile HP water or wastewater depending on the experiment. Sterile HP water or wastewater was added to no treatment and kill controls. All treatment groups and the NT controls were mixed again and incubated at room temperature for 30 minutes and the kill control was incubated at 95°C for 30 minutes. At the end of the incubation period 20 µl of 7 g/l sodium thiosulphate and 100 µl of 4.8 mM sodium bicarbonate were

added to all tubes to neutralise the reactive oxygen species and acid respectively. All samples then had 300 µl of warm DMEM (with L-glutamine and 2% FCS) added to them and each cell suspension was used to inoculate one well in a 24 well tissue culture plate containing confluent vero. Cultures were incubated as described above.

#### **3.2.3.6 Experiment 6: Virkon<sup>®</sup> S treatment**

Equal volumes of 4, 2 and 1 % v/v Virkon<sup>®</sup> S diluted in sterile HP water were added to 50 µl of *C. burnetii* suspension in 1.5 ml microfuge tubes (final concentrations were 2, 1 and 0.5% v/v respectively). Sterile HP water or wastewater was added to no treatment and kill controls. All treatment groups and the NT controls were mixed well and incubated at room temperature for 30 minutes. At the end of the incubation period 20 µl of 7 g/l sodium thiosulphate and 100 µl of 4.8 mM sodium bicarbonate were added to all tubes to neutralise the reactive oxygen species and acid respectively. All samples then had 300 µl of warm DMEM (with L-glutamine and 2% FCS) added to them and each cell suspension was used to inoculate one well in a 24 well tissue culture plate containing confluent vero. Cultures were incubated as described above.

#### **3.2.3.7 Experiment 7: Hydrogen peroxide treatment**

Equal volumes of 4, 2 and 1 % v/v H<sub>2</sub>O<sub>2</sub> diluted in sterile HP water were added to 50 µl of *C. burnetii* suspension in 1.5 ml microfuge tubes (final concentrations were 2, 1 and 0.5% v/v respectively). Sterile HP water or wastewater was added to no treatment and kill controls. All treatment groups and the NT controls were mixed well and incubated at room temperature for 30 minutes. At the end of the incubation period 20 µl of 7 g/l sodium thiosulphate was added to all tubes to neutralise the reactive oxygen species. For experiment 7.1 RNA was purified from

one set of samples and the viability assessed using a RT-qPCR as described in Chapter 4. Three hundred microliters of warm DMEM (with L-glutamine and 2% FCS) was added to each cell suspension and each suspension used to inoculate one well in a 24 well tissue culture plate containing confluent vero. Cultures were incubated as described above.

### **3.2.4 Extraction of genomic DNA from cell cultures**

Culture medium from each well was aspirated and reserved. Wells were then washed with PBS and the PBS was removed and pooled with the media from that well. All wells were trypsinised and pooled with the two previous fractions. Each sample was centrifuged at  $20,800 \times g$  for 10 minutes and whole genomic DNA was purified from the pellet using a Qiagen Tissue Minikit (Qiagen, Hilden, Germany) according to the manufacturers instructions with a 45 minute incubation at 55°C in buffer ATL plus proteinase K and a final 30 minute incubation at 70°C prior to the addition of 70% v/v ethanol (Klee, Tyczka et al. 2006). The purified DNA was eluted in 100 µl of buffer AE and the DNA concentration determined using a Nanodrop spectrophotometer (Nanodrop Technologies Wilmington USA).

### **3.2.5 TaqMan real-time PCR**

DNA samples were tested using a real-time PCR that targeted the *IS1111a* element using the TaqMan primer and probe set described in Chapter 2. Briefly, the real-time PCR reaction mixture contained 12.5 µl UDG SuperMix (Invitrogen, Mount Waverley, Victoria, Australia), 4.5 mM magnesium chloride, 25 pmoles of forward primer (IS1111aF), 20 pmoles of reverse primer (IS1111aR), 1.25 pmoles of probe (IS1111aP) and 1 µl of template. Real-time PCR assays were performed on a Rotorgene<sup>®</sup> 3000 (Corbett Life science, Mortlake, New South Wales, Australia) according to the following cycling parameters:

One hold at 50°C for two minutes, a second hold at 95°C for two minutes followed by 40 cycles of 95°C for 20 seconds and 40 seconds at 64°C.

Data were manipulated and analysed using Rotorgene® software (Corbett). All samples were run in duplicate and every run included a standard curve consisting DNA purified from Q-Vax™ vaccine (CSL, Parkville, Australia). The concentration of DNA from the Q-Vax™ vaccine was determined using a Nanodrop spectrophotometer and the number of *C. burnetii* genomes per microliter of cell suspension calculated according to the molecular weight of the *C. burnetii* genome (Coleman, Fischer et al. 2004). Serial 10-fold dilutions of the neat standard in 0.01 M TE (pH 8.0) were made. Six 10-fold dilutions of standard were included in each PCR run spanning concentrations of 18 to  $1.8 \times 10^6$  genomes per microliter. Two no template controls (NTC) were included with each run.

The Rotorgene® 3000 software was used to automatically select optimal cycle threshold cut-offs based upon the slope of the standard curve and the  $R^2$  value. The user-defined DNA concentration of the standards was then used by the software to provide estimates of the DNA concentration of the unknown samples. Microsoft Excel Professional Edition was used to format data prior to statistical analysis.

### **3.2.6 Interpretation of data**

The growth of *C. burnetii* in culture was measured using the previously described qPCR. The efficacy of each treatment was calculated as the reduction of amplifiable DNA (*C. burnetii* genomes) in each treatment group as a proportion of the untreated control wells, expressed as 'percentage reduction'.

Comparisons were only made between treated samples and no-treatment controls from the same plate to reduce variability.

### **3.2.7 Statistical analysis of data**

The statistical significance of observed differences in the percentage reduction of *C. burnetii* DNA in control and test samples from each disinfectant and between each concentration of disinfectant from the same experiments were determined using a one-way analysis of variance (ANOVA) with Tukey's test for honestly significant difference at a 95% confidence limit. Comparisons between the same treatment concentration in different experiments were made using an independent sample T-test. Analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, USA).

## **3.3 Results**

### **3.3.1 Treatment medium**

The water used was collected from the drainage sump on the Murdoch University farm and had a pH of 9.37 at 22°C, a turbidity of 73 NTU, an S/C of 1.77 at 25°C, TOC of 200 mg.C/L and TDS of 1.3 g/L. The change in pH caused by adding chemical disinfectants to HP water or wastewater at room temperature is shown in Table 3.1.

**Table 3-1. Change in pH of HP water or wastewater following the addition of a chemical disinfection agent at a temperature of 22°C**

<b>Agent</b>	<b>Concentration</b>	<b>HP water</b>	<b>Wastewater</b>
	Blank	6.17	9.37
<b>NaOCl</b>	0.0125 mM	5.91	9.35
	0.025 mM	6.75	9.35
	0.05 mM	7.45	9.35
	0.10 mM	8.32	9.35
	0.20 mM	8.73	9.35
	0.40 mM	9.16	9.38
	4.00 mM	10.52	9.43
	40.00 mM	11.56	9.64
<b>PAA</b>	3.2 x 10 <sup>-5</sup> %	4.68	9.20
	5%	1.94	2.11
	10%	1.68	1.74

	16%	1.26	1.28
<b>PAA</b>	0.0125 mM	4.14	9.17
<b>(3.2 x 10<sup>-5</sup>%) +</b>	0.025 mM	4.16	9.18
<b>NaOCl     at     3</b>			
<b>concns</b>	0.05 mM	4.10	9.20
<b>Virkon<sup>®</sup></b>	0.05%	3.05	7.44
	0.50%	2.66	5.57
	1.00%	2.37	3.41
	2.00%	2.18	2.52

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### **3.3.2 Reduction in growth of *Coxiella burnetii* exposed to sodium hypochlorite**

Results from all experiments assessing the efficacy of disinfection with NaOCl are shown in Table 3.2 and the kill controls used for each experiment are shown in Table 3.3.

**Table 3-2. Reduction in growth of *Coxiella burnetii* (% red) after exposure to NaOCl at varying concentrations, exposure times and in different mediums measured using a qPCR**

Exp.	Concentration		Exposure	% red.	SEM red	%
	n	Treated in				
<b>1.1</b>	40 mM	PBSS	30 min	95.41	0.18	
	4 mM	PBSS	30 min	95.21	0.23	
	0.4 mM	PBSS	30 min	92.87	0.73	
<b>1.2</b>	0.4 mM	PBSS	30 min	98.32	0.59	
	0.2 mM	PBSS	30 min	97.57	0.24	
	0.1 mM	PBSS	30 min	92.38	1.16	
<b>1.3</b>	0.2 mM	Wastewater	30 min	93.33	0.80	
	0.1 mM	Wastewater	30 min	94.10	0.27	
	0.05 mM	Wastewater	30 min	90.42	0.64	
<b>1.4</b>	0.05 mM	PBSS	5 min	41.62	4.52	
	0.05 mM	PBSS	10 min	43.37	7.38	

0.05 mM	PBSS	20 min	72.70	2.21
0.05 mM	PBSS	30 min	70.87	3.95

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**Table 3-3. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with each NaOCl disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>1.1</b>	300mJ UV	80.08	1.24
<b>1.2</b>	20mM NaOCl	99.95	0.02
<b>1.3</b>	20mM NaOCl	96.63	0.11
<b>1.4</b>	30min @95°C	98.90	0.48

The percentage reduction in amplifiable *C. burnetii* DNA measured for the no treatment controls (NT) in experiment 1.1 was significantly less than all treatment groups and the kill controls ( $p < 0.001$ ). The percentage reduction in amplifiable DNA in the “kill” controls was significantly greater than all treatment groups ( $p < 0.05$ ) except for the 0.4 mM NaOCl-treated cells ( $p = 0.061$ ). The treatment groups did not yield results that were statistically different from each other ( $p > 0.9$ ). When a comparison was made between the samples treated with 0.4 mM NaOCl in experiment 1.1 and the same concentration in experiment 1.2 the percentage reduction in DNA was found to be significantly greater in experiment 1.2 ( $p < 0.001$ ). The percentage reduction in amplifiable DNA for the no treatment controls (NT) was significantly less than all treatment groups and the kill controls ( $p < 0.001$ ) and the kill controls had significantly greater reduction in DNA than the 0.1 mM NaOCl-treated cells ( $p = 0.061$ ) in experiment 1.2. The percentage reduction of amplifiable DNA in the treatment groups within experiment 1.2 were not statistically different from each other ( $p > 0.9$ ).

There was significantly less reduction in amplifiable DNA when *C. burnetii* was exposed to 0.2 mM NaOCl in wastewater compared to (experiment 1.3) PBSS (experiment 1.2) ( $p < 0.001$ ). There was no significant difference in the amplifiable DNA when *C. burnetii* was exposed to 0.1 mM NaOCl in wastewater or PBSS. There was significantly less reduction in amplifiable DNA in *C. burnetii* not exposed to NaOCl (NT controls) in wastewater compared to all other treatments (experiment 1.3) ( $p < 0.001$ ). There was no significant difference between the reduction in amplifiable DNA in each of the treatment groups or the “kill” controls suspended in wastewater.

When the efficacy of 0.05 mM NaOCl was examined over time it was found that there was significantly less reduction in amplifiable DNA in the NT controls

compared to the “kill” controls ( $p<0.001$ ) and significantly less amplifiable DNA in cells exposed to 0.05 mM NaOCl for each incubation period ( $p<0.05$ ). There was significantly greater reduction in amplifiable DNA in the “kill” controls compared to cells exposed to 0.05 mM NaOCl for each incubation period ( $p<0.05$ ). There was significantly less reduction in amplifiable DNA in cells exposed to 0.05 mM NaOCl for five and 10 minute compared to cells exposed for 20 and 30 minute incubations ( $p<0.05$ ).

### **3.3.3 Reduction in growth of *Coxiella burnetii* exposed to UV radiation**

Results from experiments to assess the efficacy of disinfection with UV radiation are shown in Table 3.4. The results from the “kill” controls used in each experiment are shown in table 3.5.

**Table 3-4. Reduction in growth of *Coxiella burnetii* (% red) after exposure to UV at varying doses and in two cell suspension mediums using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<hr/>					
<b>2.1</b>	40				
	mJ/cm <sup>2</sup>	HP Water	NA	94.00	0.63
	10				
	mJ/cm <sup>2</sup>	HP Water	NA	94.67	0.21
	1 mJ/cm <sup>2</sup>	HP Water	NA	61.83	8.49
<b>2.2</b>	40				
	mJ/cm <sup>2</sup>	Wastewater	NA	54.32	3.96
	10				
	mJ/cm <sup>2</sup>	Wastewater	NA	51.55	1.73
	1 mJ/cm <sup>2</sup>	Wastewater	NA	28.40	8.01
<b>2.3</b>	10				
	mJ/cm <sup>2</sup>	HP Water	NA	95.22	0.56
	5 mJ/cm <sup>2</sup>	HP Water	NA	77.07	3.74

1 mJ/cm <sup>2</sup>	HP Water	NA	29.02	23.47
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**Table 3-5. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with each UV radiation disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>2.1</b>	3 M NaOH	96.83	0.20
<b>2.2</b>	100 mJ/cm <sup>2</sup>	73.82	2.90
<b>2.3</b>	200 mJ/cm <sup>2</sup>	99.67	0.04

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The percentage reduction in amplifiable DNA in the no treatment controls (NT) was significantly lower than all treatment groups and the “kill” controls in experiment 2.1 ( $p<0.001$ ). There was significantly lower reduction in amplifiable DNA in the samples exposed to 1 mJ/cm<sup>2</sup> of UV radiation compared to the “kill” controls and samples exposed to 10 and 40 mJ/cm<sup>2</sup> of UV radiation ( $p<0.05$ ). There was no significant difference in the reduction of amplifiable DNA in samples exposed to 10 and 40 mJ/cm<sup>2</sup> compared to the “kill” controls and compared to each other.

In experiment 2.2 there was significantly lower percentage reduction in amplifiable DNA in the no treatment control (NT) samples compared to all treatment groups and the “kill” controls ( $p<0.002$ ). There was significantly greater reduction in amplifiable DNA in the “kill” control samples compared all treatment groups ( $p<0.05$ ). There was significantly greater reduction in amplifiable DNA in samples exposed to 10 and 40 mJ/cm<sup>2</sup> of UV radiation compared to samples exposed to 1 mJ/cm<sup>2</sup> and the kill controls ( $p<0.05$ ). There was no significant difference in the percentage reduction in amplifiable DNA in samples exposed to 10 mJ/cm<sup>2</sup> compared to sample exposed to 40 mJ/cm<sup>2</sup> of UV radiation

In experiment 2.3 the percentage reduction in DNA in the no treatment control (NT) samples was significantly less than in samples from all treatment groups except those exposed to 1 mJ/cm<sup>2</sup> of UV radiation and the “kill” controls ( $p<0.003$ ). There was significantly greater reduction in amplifiable DNA in “kill” control samples compared to samples exposed to 1 mJ/cm<sup>2</sup> of UV radiation ( $p<0.01$ ). There was a significantly greater reduction in amplifiable DNA in samples exposed to 10 mJ/cm<sup>2</sup> compared to samples exposed to 1 mJ/cm<sup>2</sup> UV radiation ( $p<0.001$ ).

There was significantly less reduction in amplifiable DNA in samples suspended in wastewater and exposed to UV radiation compared to samples suspended in PBSS and exposed to UV radiation ( $p<0.001$ ).

#### **3.3.4 Reduction in growth of *Coxiella burnetii* exposed to ozone**

Results from experiments assessing the efficacy of disinfection with ozone are shown in Table 3.6. Results from the “kill” controls used in each experiment are shown in Table 3.7.

**Table 3-6. Reduction in growth of *Coxiella burnetii* (% red) after exposure to ozone at varying concentrations and in two mediums using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<b>3.1</b>	43 mg/L	PBSS	10 min	100.00	0.00
	43 mg/L	PBSS	20 min	100.00	0.00
	43 mg/L	PBSS	30 min	100.00	0.00
<b>3.2</b>	12 mg/L	PBSS	1 min	3.50	25.25
	12 mg/L	PBSS	5 min	-84.67	50.42
	12 mg/L	PBSS	10 min	64.17	9.99
<b>3.3</b>	12 mg/L	Wastewater	1 min	-52.58	59.49
	12 mg/L	Wastewater	5 min	17.38	2.42
	12 mg/L	Wastewater	10 min	16.00	5.48

**Table 3-7. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with each ozone disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>3.1</b>	3 M NaOH	97.87	0.48
<b>3.2</b>	15 min in 12 mg ozone	98.67	0.49
<b>3.3</b>	100 mJ/cm <sup>2</sup>	66.85	4.87

There was significant variation in the results obtained from exposing *C. burnetii* cells to ozone. In experiment 3.1 there was significantly less reduction in amplifiable DNA in the NT control samples compared to all other treatment groups and the “kill” controls ( $p<0.001$ ). In experiment 3.2 there were no significant differences in amplifiable DNA between any treatment or control group except that there was significantly less reduction in amplifiable DNA in cells exposed to ozone for 5 minutes compared to cells exposed for 10 minutes treatment group and the “kill” control group ( $p<0.005$ ).

In experiment 3.3 there was significantly less reduction in DNA in cells suspended in wastewater and exposed to ozone for one minute exposure compared to cells suspended in wastewater and exposed to ozone for 10 minutes and the “kill” control samples ( $p<0.05$ ). There was significantly greater reduction in amplifiable DNA in samples suspended in pure buffer and exposed to 12 mg/L ozone for 10 minutes compared to samples suspended in wastewater and exposed to 12 mg/L ozone for 10 minutes ( $p<0.005$ ).

### **3.3.5 Reduction in growth of *Coxiella burnetii* exposed to peracetic acid**

Results from experiments assessing the efficacy of disinfection with peracetic acid are shown in Table 3.8. The results from the “kill” controls used in each experiment are shown in Table 3.9.

**Table 3-8. Reduction in growth of *Coxiella burnetii* (% red) after exposure to peracetic acid at varying concentrations in HP water using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<b>4.1</b>	16%	HP Water	60 min	-150.70	24.15
	10%	HP Water	60 min	-40.43	32.25
	5%	HP Water	60 min	-10.65	12.67

**Table 3-9. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with the peracetic acid disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>4.1</b>	3 M NaOH	81.85	3.08

In comparison to all other treatment groups and both controls, treatment with 16% PAA resulted in a significant increase in replication of *C. burnetii* cells ( $p<0.05$ ). That is; the samples treated with 16% PAA were observed to have 50% more *C. burnetii* DNA in them than the NT controls. The only other significant differences observed in this experiment were between the kill control, the 5% PAA treated group ( $p<0.05$ ) and the 10% PAA treated samples ( $p<0.01$ ), with the kill control resulting in significantly greater reduction of DNA in both cases.

### **3.3.6 Reduction in growth of *Coxiella burnetii* exposed to peracetic acid in combination with hypochlorite**

Results from experiments assessing the efficacy of disinfection with PAA used in combination with NaOCl are shown in Table 3.10. Results from the “kill” controls used in each experiment are shown in Table 3.11.



**Table 3-10. Reduction in growth of *Coxiella burnetii* (% red) after exposure to  $3.2 \times 10^{-5}\%$  peracetic acid combined with hypochlorite at varying concentrations in HP water and in wastewater using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<b>5.1</b>	0.05 mM	HP Water	60/30 min	99.93	0.04
	0.025 mM	HP Water	60/30 min	99.60	0.05
	0.0125 $\mu$ mM	HP Water	60/30 min	98.68	0.14
	PAA only	HP Water	60/30 min	97.82	0.22
<b>5.2</b>	0.05 mM	Wastewater	60/30 min	-76.57	20.21
	0.025 mM	Wastewater	60/30 min	-21.53	10.64
	0.0125 $\mu$ mM	Wastewater	60/30 min	-7.13	16.90
	PAA only	Wastewater	60/30 min	-57.08	19.95

**Table 3-11. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with each PAA/NaOCl disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>5.1</b>	30min @95°C	99.93	0.02
<b>5.2</b>	30min @95°C	97.78	0.10

There was significantly less reduction in amplifiable DNA in cells suspended in HP water and exposed to a combination of PAA and NaOCl compared to the NT control samples and samples in all other test and control groups ( $p<0.001$ ). There was no significant difference in the reduction of amplifiable DNA in samples treated with PAA alone and PAA in combination with NaOCl.

There was significantly greater reduction of amplifiable DNA in the kill control samples suspended in wastewater compared to all treatment groups and the NT controls suspended in wastewater ( $p<0.001$ ). There was significantly less reduction in amplifiable DNA in samples suspended in wastewater and exposed to  $3.2 \times 10^{-5}\%$  PAA in combination with 0.05 mM NaOCl compared to the NT control suspended in wastewater ( $p<0.05$ ) and the samples suspended in wastewater and exposed to PAA and 0.0125 mM NaOCl ( $p<0.05$ ). There was significantly greater reduction of amplifiable DNA in samples that had been suspended in HP water for all treatment groups and the kill controls compared to samples suspended in wastewater ( $p<0.005$ ). There was no significant difference between the reduction in amplifiable DNA in the NT controls suspended in HP water and wastewater ( $p=1$ ).

### **3.3.7 Reduction in growth of *Coxiella burnetii* exposed to Virkon® S**

Results from experiments assessing the efficacy of disinfection with Virkon S® are shown in Table 3.12. Results from the “kill” controls used in each experiment are shown in Table 3.13.

**Table 3-12. Reduction in growth of *Coxiella burnetii* (% red) after exposure to Virkon<sup>®</sup> S at varying concentrations in HP water and in wastewater using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<b>6.1</b>	2.00%	HP Water	30 min	99.93	0.02
	1.00%	HP Water	30 min	99.38	0.16
	0.50%	HP Water	30 min	99.44	0.10
	0.05%	HP Water	31 min	97.27	0.52
<b>6.2</b>	2.00%	Wastewater	30 min	99.8	0.01
	1.00%	Wastewater	30 min	99.75	0.02
	0.50%	Wastewater	30 min	99.33	0.04
	0.05%	Wastewater	30 min	96.00	0.23

**Table 3-13. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with each Virkon<sup>®</sup> S disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>6.1</b>	3 M NaOH	97.77	0.96
<b>6.2</b>	30min @95°C	99.88	0.02

There was significantly greater reduction in amplifiable DNA in samples all treatment and “kill” control groups compared to the NT controls suspended in HP water and waster water ( $p<0.001$ ). There was significantly greater reduction in amplifiable DNA in cells suspended in HP water and exposed to 2% w/v Virkon S<sup>®</sup> compared to cells suspended in wastewater and exposed to 2% w/v Virkon S<sup>®</sup> ( $p<0.005$ ).

### **3.3.8 Reduction in growth of *Coxiella burnetii* exposed to hydrogen peroxide**

The results from all experiments assessing the efficacy of disinfection with H<sub>2</sub>O<sub>2</sub> are shown in table 3.14 and the kill controls used in each experiment are shown in table 3.15.

**Table 3-14. Reduction in growth of *Coxiella burnetii* (% red) after exposure to hydrogen peroxide at varying concentrations in HP water using a qPCR**

<b>Exp.</b>	<b>Conc.</b>	<b>Treated in</b>	<b>Exposure</b>	<b>% red.</b>	<b>SEM (% red.)</b>
<b>7.1</b>	2.00%	HP Water	30 min	90.78	1.06
	1.00%	HP Water	30 min	90.73	0.65
	0.50%	HP Water	30 min	90.30	0.83

**Table 3-15. The reduction in growth of *Coxiella burnetii* (% red) in “kill” controls used with the hydrogen peroxide disinfection experiment**

<b>Exp.</b>	<b>Kill control</b>	<b>Kill Ctrl % red.</b>	<b>SEM (Kill Ctrl)</b>
<b>7.1</b>	20mM NaOCl	99.85	0.03



There was significantly greater reduction in amplifiable DNA in cells exposed to all concentrations of H<sub>2</sub>O<sub>2</sub> and the “kill” control compared to cells in the NT control groups ( $p < 0.001$ ). There was significantly less reduction in amplifiable DNA in samples exposed to 0.5 % H<sub>2</sub>O<sub>2</sub> compared to the “kill” control, 1 % H<sub>2</sub>O<sub>2</sub> and 2 % H<sub>2</sub>O<sub>2</sub> groups ( $p < 0.005$ ). There was no significant difference in the reduction of amplifiable DNA in samples exposed to 1 % and 2% H<sub>2</sub>O<sub>2</sub> and between either of these groups and the “kill” control ( $p = 1$ ).

### **3.4 Discussion**

#### **3.4.1 Controls**

The physical and chemical resistance of *C. burnetii* was highlighted in this series of experiments by the difficulty that was experienced in finding a treatment that, when applied to viable cells, could achieve complete inactivation. Treatment with a strong base (NaOH) achieved only partial inactivation of *C. burnetii* and high doses of UV radiation produced variable results. Treatment with heat or relatively concentrated NaOCl proved to be the most reliable and effective of the “kill” controls used. However, chronologically these treatments were only used in the later experiments. Ransom and Huebner (1951) showed that heat inactivation of *C. burnetii* was a valid disinfection method but it is probably not viable on a commercial scale because of the energy required to attain sufficiently high temperatures in large volumes of effluent.

#### **3.4.2 Sodium hypochlorite**

Sodium hypochlorite is the most widely used chlorine-releasing agent (CRA) in disinfection applications (McDonnell and Russell 1999) but it has previously been shown to have limited effectiveness for inactivating *C. burnetii* (Scott and Williams

1990). The results from this study indicate that phase II *C. burnetii* is highly sensitive to treatment with NaOCl at a concentration approximately 100 times more dilute than that used by Scott and Williams (1990) and with a much shorter contact time. The disparity between our results and those of Scott and Williams (1990) may be due to the method used in the earlier study to assess viability of *Coxiella*. Scott and Williams (1990) used mouse inoculation and proposed that the presence of anti-*C. burnetii* antibodies was evidence that the inoculum contained viable organisms. This is a flawed assumption as animals will produce an immune response to foreign cells regardless of their viability. Additionally, this method of evaluating viability is likely to be less reproducible than the tissue culture assay described here because the host immune response to challenge with *C. burnetii* can be unpredictable (Berri, Souriau et al. 2002).

The addition of NaOCl to pure water maintained a pH in the proposed effective range up to a concentration of 0.025 mM. However, the addition of NaOCl to wastewater had a negligible affect on the starting pH (9.37) across the whole hypochlorite concentration range tested. Previous studies have shown that NaOCl is most effective in a pH range of 4-7 where hypochlorous acid species predominate (McDonnell and Russell 1999). Hypochlorous acid has been shown to cause a range of biocidal effects including cell membrane damage and susceptibility to subsequent stress (Cortezzo, Koziol-Dube et al. 2004), DNA damage via the formation of the highly reactive singlet oxygen and inhibition of enzyme action (Mokgatla, Gouws et al. 2002). However, at the concentrations used in this study inhibition of protein, RNA and DNA synthesis, and consequently cell division, appear to be the most likely affects (McKenna and Davies 1988). In high pH conditions (above 9.0) the hypochlorite ion predominates but it is thought that this chemical species does not significantly contribute to disinfection (McDonnell and Russell 1999). Hypochlorite ions are naturally produced in

monocytes following phagocytosis (Clark 1990; Hawkins and Davies 1998), which may serve as a biological trigger for cellular differentiation for intracellular pathogens such as *C. burnetii* whose reproductive niche is the phagolysosome.

The results of these experiments show that there was significantly less inactivation of *C. burnetii* cells exposed to 0.2 mM NaOCl in wastewater compared to HP water. This suggests that organic matter in the wastewater inhibited the disinfectant action, which is in agreement with past studies (Scott and Williams 1990). However, no significant differences were observed in the viability of *Coxiella* exposed to 0.1 mM NaOCl in wastewater compared to HP water, which could indicate that the chemical equilibrium reached with a lower concentration of NaOCl was more favourable to the inactivation process. Overall it appears that sodium hypochlorite treatment is an appropriate disinfectant for a laboratory and, more importantly, retains its efficacy in water contaminated with organic matter.

### **3.4.3 Sodium hypochlorite in combination with peracetic acid**

A combination of peracetic acid and hypochlorite was evaluated because it was assumed that NaOCl would have higher disinfectant efficacy in acidic conditions. Peracetic acid in combination with NaOCl maintained an approximate pH of 4 in HP water which is in the optimal range for hypochlorous acid formation. However, the same combination of disinfectants in wastewater did not reduce the pH below 9.1. There was close to 100% retardation of *C. burnetii* growth in samples exposed to each NaOCl concentrations in HP water. However, in wastewater and PAA alone, or in combination with NaOCl, there appeared to be an enhancement in the replication of coxiellae. While PAA is considered to be a more effective biocide than hydrogen peroxide it functions in much the same way and has similar limitations relating to efficacy in solutions with organic content.

Both act as oxidants and generate OH radicals which attack lipids, proteins and DNA but high concentrations and contact times are often required, particularly in the presence of organic matter (Baldry, French et al. 1991; McDonnell and Russell 1999; Koivunen and Heinonen-Tanski 2005). The apparent dose-dependent increase in replicative ability of *C. burnetii* cells treated with PAA in wastewater is difficult to explain given its proposed mode of action. One possible explanation is that the quenching effect of organic compounds in the wastewater combined with the relatively high pH reduced the concentration of reactive peroxide species (Baldry 1983) to a level that was sub-inhibitory. This may have replicated the respiratory burst and subsequent peroxide formation that takes place in the lysosomes of phagocytic cells (McKenna and Davies 1988; Clark 1990). This may serve as a trigger for initiation of cell differentiation of *C. burnetii* into the metabolically active LCV and thus decrease the lag period before exponential division can occur in host cells (Coleman, Fischer et al. 2004). Moreover, the combined use of these agents did not appear to produce a synergistic affect in pure water. In wastewater the results of using NaOCl and PAA in combination are more difficult to interpret and suggest that this combination does not provide a disinfection solution able to inactivate *C. burnetii* in agricultural effluent.

#### **3.4.4 Ultra-violet radiation**

The results from this study support earlier work that showed that *C. burnetii* was susceptible to inactivation by ultra-violet radiation (Ransom and Huebner 1951; Little, Kishimoto et al. 1980). This study provides new data with regards the end-point of the effectiveness of UV exposure. However, it appears that it is difficult to achieve complete inactivation using UV radiation. Indeed, even relatively high doses of radiation left approximately five percent of cells viable when treatment was performed in pure water. Using UV radiation to inactivate *C.*

*burnetii* in wastewater was found to be significantly less effective than in pure water. These results are not surprising and support other work suggesting that cell clumping, shadowing and aggregation within suspended solids can protect microorganisms from this method of disinfection (Hill, Kantardjieff et al. 2002; Koivunen and Heinonen-Tanski 2005). Commercial scale treatment of effluent with UV radiation is typically done with a dose of approximately 30-40 mJ/cm<sup>2</sup> (Shin, Linden et al. 2001). This study showed that only approximately 50% of *C. burnetii* cells were inactivated at this level of irradiation when treatment was undertaken in wastewater. Therefore, significant pre-treatment of wastewater would be required prior to administering UV treatment if inactivation of *C. burnetii* was a primary aim for the treatment of effluent.

While UV treatment does not form dangerous by-products and its efficacy is independent of factors such as pH and temperature (Quek and Hu 2008) some organisms are able to reverse the damage done by this form of radiation. Ultra violet radiation causes the formation of pyrimidine dimers in the organisms DNA which prevents replication (Rubin, Menshonkova et al. 1981) but these dimers can be repaired (Shin, Linden et al. 2001; Quek and Hu 2008) allowing the normal cellular division to occur. A recent study by Mertens, Lantsheer and Samuel (Mertens, Lantsheer et al. 2005) found that while certain genetic elements commonly associated with DNA repair after oxidative stress were absent in the *C. burnetii* genome, the organism displays a fully functional nucleotide excision repair system. Therefore, *C. burnetii* may be able to excise and repair pyrimidine dimers caused by UV radiation. If UV irradiation were to be considered as a method for inactivating *C. burnetii* it would first be necessary to evaluate this organism's capability for repairing pyrimidine dimers.

### 3.4.5 Ozone

The disinfectant efficacy of ozone was inconsistent in this study. This was probably due to the simple nature of the apparatus used to generate and dispense ozone. It may have been possible to obtain better results with a more precise delivery mechanism and accurate concentration control. The experiments seemed to indicate that ozone was very effective at inactivating *C. burnetii* at high concentrations but no end point dose could be determined because of limitations in the equipment used. However, ozone is a strong oxidising agent, which can generate dangerous by-products when used in high concentrations in water containing bromide or iodide (von Gunten 2003). Therefore, treatment of water with this agent must be considered carefully before being implemented.

### 3.4.6 Virkon<sup>®</sup> S

Treatment of *C. burnetii* cells with Virkon<sup>®</sup> S produced significant inactivation at all concentrations used in both pure water and wastewater. This suggests that Virkon<sup>®</sup> S may be appropriate for small-scale use in an agricultural or laboratory setting. Virkon<sup>®</sup> S is a commercially available oxidative disinfectant that is purported to contain a balanced, stabilised blend of peroxygen compounds, surfactant, organic acids and an inorganic buffer system, which in solution is activated to form hypochlorous acid (DuPont 2008). The mode of action of Virkon<sup>®</sup> S will thus be similar to the other oxidants described previously. However, the combination of these oxidants with a surfactant in a stable composition may produce greater bactericidal action in a variety of conditions. Virkon<sup>®</sup> S appeared to maintain its activity in the presence of organic matter and suspended solids. However, the efficacy of Virkon<sup>®</sup> S has been shown to be variable in conditions of high organic load (McCormick and Maheshwari 2004). The low pH that resulted from adding Virkon<sup>®</sup> S to both of the water types used in these experiments is

likely to have played a significant role in the disinfection efficacy observed, particularly in relation to hypochlorous acid. It appears that the resistance of *C. burnetii* to the normally peroxide-rich host cell phagolysosome (Clark 1990) is attributed to inhibition of the respiratory burst by acid phosphatase activity (Baca, Roman et al. 1993). In light of the metabolic effort *C. burnetii* assigns to inhibit host cells production of peroxide, peroxygens may be ideal candidate disinfectants. However, Virkon<sup>®</sup> S is becoming more difficult to obtain in Australia and its cost is prohibitive for large-scale use. Therefore, its use may only be applicable to laboratories and small volumes of animal-associated effluent.

### 3.4.7 Hydrogen peroxide

Hydrogen peroxide produced a reasonable reduction in the growth of *C. burnetii* at all the concentrations assessed in this experiment. This agent acts as an oxidant and generates hydroxyl radicals, which are extremely effective biocides but it is considered to be a less effective bactericide than other oxidising agents (Baldry 1983; McDonnell and Russell 1999). Hydrogen peroxide is also relatively unstable and requires higher concentrations to achieve penetration through a medium with high organic load (Hawkins and Davies 1998) and thus it has relatively little use as a biocide (Baldry 1983). Hydrogen peroxide is not a potential candidate for disinfection of wastewater.

### 3.4.8 Conclusions

Application of the cell culture–PCR method to assess the efficacy of disinfectants identified several candidates that may be used to inactivate *C. burnetii* in wastewater in commercial settings. In particular, sodium hypochlorite appears to be an attractive option. The technology for administering this disinfectant is already established and could be adopted immediately for *C.*

*burnetii*-specific use. However, wastewater treated with sodium hypochlorite may only be compatible with re-use in certain situations where the potential toxic effects of chlorination by-products are not an issue.

It is important to note that the data presented in this study showed that none of the agents were able to completely inactivate *C. burnetii*. This is likely to pose a problem for re-use of wastewater that potentially contains *C. burnetii* because of the low infectious dose of the organism (Tigertt, Benenson et al. 1961; Ormsbee, Peacock et al. 1978). It is not likely to be feasible to achieve complete inactivation of *C. Burnetii* in dynamic waste streams with the large volumes of water found in agricultural industries. This is mainly due to the 'tailing' phenomenon, which is characterised by a linear decrease in viable organisms remaining in response to exponential increases in disinfectant concentration (Koivunen and Heinonen-Tanski 2005). Instead disinfection should be considered to be part of a broader risk reduction process that takes into account the dilution and settling effects that may greatly reduce the concentration of pathogens such as *C. burnetii* in the end-point effluent.



## **4. A RT-qPCR for detection of viable *Coxiella burnetii* cells in environmental matrices**

### **4.1 Introduction**

The need for bacterial viability assays has led to the development of new methods for discrimination between 'live' and 'dead' pathogens. Several fluorescence-based assays have been developed, mostly aimed at detecting respiratory activity (Bhupathiraju, Hernandez et al. 1999), membrane potential (Deere, Porter et al. 1995) and membrane integrity (Nebe-von-Caron, Stephens et al. 2000) in viable cells. Some researchers consider that all of the assays described above must be performed to adequately assess viability of pathogenic microorganisms (Laflamme, Lavigne et al. 2004). However, performing all of these tests is both time consuming and expensive. Therefore, more unequivocal tests are required for routine monitoring and surveillance applications.

The viability of *C. burnetii* has been estimated using plaque assays (McDade and Gerone 1970; Ormsbee, Peacock et al. 1978; Schneider 1989) similar to those used to detect the viability of viruses. However, *C. burnetii* is relatively slow growing and the assays may take more than 10 days to complete and may produce variable results (Cory, Yunker et al. 1974; Schneider 1989). In addition, the results from plaque assays are not always comparable with results from experimental infection of laboratory animals and are not always analogous to titrations in animals (Wike, Tallent et al. 1972).

Therefore, animal inoculation remains the gold standard test to determine the viability of *C. burnetii* (Arricau-Bouvery, Souriau et al. 2005). However, the method has limitations in terms of the time required, expense, consistency of results and

ethical issues concerning treatment of animals (Ransom and Huebner 1951; Malloch and Stoker 1952; Scott and Williams 1990; Sobsey and Leland 2001). Furthermore, animal infection procedures have not been applied directly to environmental samples which have high organic and microbial loads. Similarly, the cell culture-qPCR system described in Chapter 3 (Shin, Linden et al. 2001; Rochelle, Marshall et al. 2002; Brennan and Samuel 2003) would require significant modification before it could be used to test soil or faecal samples.

Detection of transcriptional activity to verify the viability of a pathogen is an established technique (Stinear, Matusan et al. 1996; Jenkins, Trout et al. 2003). However, the technique cannot be directly applied to organisms such as *C. burnetii*, which can remain viable but metabolically dormant in the environment for months or longer without significant modification (PHAC 2001). Alternative approaches have been applied to detect the viability of *Cryptosporidium*, which is similar to *C. burnetii* in that it is an obligate intracellular parasite with a high level of environmental stability. Attempts have been made to use reverse transcriptase PCR to detect the transcribed RNA population for heat shock protein following heat treatment of *C. parvum* oocysts (Fontaine and Guillot 2003). Hackstadt and Williams (1981) showed that the metabolic activity, and hence RNA transcription, of *C. burnetii* was stimulated by exposing the organism to acidic conditions. However, it would not be feasible to use this method with environmental samples because of the time taken to test and adjust the pH of a variety of environmental substrates and the increased risk of cross-contamination of samples. Therefore, heat treating environmental samples prior to RNA extraction and subsequent detection by quantitative PCR may be the most robust and sensitive method for detecting and viable *C. burnetii* cells in substrates such as soil.

## 4.2 Materials and methods

### 4.2.1 Culture and extraction of *Coxiella burnetii* from tissue culture cells

Vero (African green monkey kidney) cells infected with *C. burnetii* Phase II Nine Mile RSA439 (clone 4) were grown in 25 cm<sup>2</sup> culture flasks (TPP, Switzerland) at 37°C with 5% CO<sub>2</sub> (v/v) in DMEM (MP Biomedicals, Australia) supplemented with 200mM L-Glutamine (MP Biomedicals, Australia) and 2% v/v foetal calf serum. Cultures were grown without replenishment of media for approximately four weeks to maximise the proportion of SCV to LCV (Coleman, Fischer et al. 2004). The residual monolayer was manually scraped from the flask surface and homogenised in cell culture medium. The cell suspension was transferred to a 40 ml centrifuge tube and centrifuged at 40,000 × *g* for 10 minutes. The cell pellet was resuspended in sterile MilliQ water to induce osmotic shock and passed through a 25 gauge needle 10 times to free coxiellae from host cell components and lyse any remaining LCV's. *Coxiella* cells were purified from host cell debris using differential centrifugation according to Baca *et al.* (Baca, Aragon et al. 1981), which consisted of three cycles of low speed (820 *g*) centrifugation for 10 minutes followed by high speed (20,800*g*) centrifugation for 10 minutes. The final cell pellet was resuspended in PBS (pH 7.4) containing 0.25M sucrose. The concentration of *C. burnetii* cell suspensions were adjusted to approximately 23,000 +/- 2,000 *C. burnetii* cells per microliter according to the method used by Baca *et al.* (1981) and 50 µl aliquots were dispensed into 1.5 ml microfuge tubes and stored at 4°C until required.

### 4.2.2 Heat treatment of cells

A total of 48 aliquots of the purified *C. burnetii* cell suspensions were placed in separate heating blocks set at 37, 40, 44 and 48°C. Three tubes were removed

from each heating block after 10, 20, 30 and 40 minutes. A further 3 aliquots of purified *C. burnetii* cell suspensions were left at room temperature to serve as unheated controls.

#### **4.2.3 Extraction and purification of RNA from cell culture-derived *Coxiella burnetii***

Whole cellular RNA was extracted from each heat-treated and untreated *C. burnetii* cell suspension using TRI Reagent according to the manufacturer's instructions (Ambion, Inc., Austin, Texas, USA). The final suspension of RNA was made in 89 µl of RNase-free water containing 10 µl of Turbo DNA-free 10x buffer and 1 µl of Turbo DNA-free DNase (Ambion). Samples were mixed gently and then incubated at 37°C for 45 minutes. The DNase was removed following DNA digestion with Turbo DNA-free inactivation reagent (Ambion) according to the manufacturer's instructions and purified using the RNA cleanup protocol described in the Qiagen RNeasy Minikit (Qiagen, Hilden, Germany). RNA was eluted from the spin column membrane with 50 µl of RNase-free water. To maximise the yield of RNA the first eluate was returned to the spin column and the column was centrifuged a second time, then the RNA sample was quantified as described below.

#### **4.2.4 Reverse transcription Polymerase Chain Reaction (RT-PCR)**

The concentration of RNA in each of the samples was measured using the Nanodrop spectrophotometer (Nanodrop Technologies Wilmington USA). A total of 75 ng of RNA was used for each reverse transcription reaction to produce complimentary DNA (cDNA) using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) as per the manufacturer's instructions. Reverse transcription reactions without reverse transcriptase enzyme were made

for all samples to ensure that no DNA had been carried over during the purification procedure.

#### **4.2.5 Preparation of soil samples containing *Coxiella burnetii***

Soil was collected from cattle holding yards on the Murdoch University farm (Murdoch Drive, Murdoch, Western Australia). Two hundred micrograms (0.2g) of soil was added to each of 12 bead-beating tubes from a MO BIO Powersoil DNA Isolation Kit (MO BIO Laboratories, Solana Beach, California, USA) from which beads and bead solution had been removed and reserved. Ten microliters of purified DNA extracted from a single dose of Q Vax™ vaccine (CSL, Parkville, Australia), containing an estimated concentration of 180,000 genomes per microliter, was added to each of nine bead beating tubes containing soil. Ten microliters of sterile water was added to each of 3 further tubes as controls.

Two grams of soil was added to 21 bead beating tubes from a MO BIO Power Soil RNA Isolation kit from which the beads had been removed. Fifty microliters of a cell suspension containing approximately  $1 \times 10^6$  cell culture-derived *C. burnetii* cells was added to 18 bead beating tubes containing soil and 18 bead-beating tubes not containing soil and vortexed to mix. The *C. burnetii* cells were grown in tissue culture and purified as described previously. The absorbance value of the purified cells at 250nm was used to adjust the concentration of the cell suspension such that each 50  $\mu$ l contained approximately  $1 \times 10^6$  cells as described in Chapter 2. Fifty microliters of sterile water was added to 3 bead-beating tubes and kept as controls. All sample tubes were stored in the dark at room temperature until required.

#### **4.2.6 Extraction of genomic DNA from soil samples**

Every seven days the reserved beads and bead solution were returned to each of three tubes containing soil and *C. burnetii* DNA. Whole genomic DNA was then purified from each tube using the MO BIO PowerSoil DNA Isolation Kit according to the manufacturer's instructions.

#### **4.2.7 Extraction and reverse transcription of RNA from soil samples**

Every seven days three tubes containing soil and three tubes without soil were heated at 40°C for 20 minutes to stimulate transcription. The reserved beads were returned to each tube and the RNA was purified immediately. On the first occasion RNA was also extracted from the water-only controls. The standard MO BIO RNA purification protocol was followed and the final RNA pellet was resuspended in 86 µl of RNase-free water containing 10 µl of Turbo DNA-free 10x buffer and 2 µl of Turbo DNA-free DNase enzyme (Ambion). Samples were mixed gently and then incubated at 37°C for 30 minutes followed by the addition of a further 2 µl of DNase enzyme. The tubes were again mixed gently and incubated at 37°C for another 30 minutes. Following DNA digestion DNase was removed using Turbo DNA-free inactivation reagent according to the manufacturer's instructions (Ambion) and subsequently purified using the RNA cleanup protocol described in the Qiagen RNeasy Minikit (Qiagen). Elution of RNA from the spin column membrane was performed using 50 µl of RNase-free water. To maximise the yield of RNA the first eluate was returned to the spin column and centrifuged a second time and then the concentration of RNA in each sample was measured using the Nanodrop spectrophotometer. Ten micrograms of RNA was used for each reverse transcription reaction using the High Capacity cDNA Archive Kit. Seventy five nanograms of total RNA from soil free of *C. burnetii* were subjected to reverse transcription. Reverse transcription reactions without reverse

transcriptase enzyme were performed for all samples to ensure that no DNA was carried over during the purification procedure. Reverse transcription was performed using an Applied Biosystems GeneAmp® PCR System 2700 according to the High Capacity cDNA Archive Kit instructions.

#### **4.2.8 Blocking of RNA Transcription with Rifampicin**

*Coxiella burnetii* cells were grown and purified as described previously. The concentration of *C. burnetii* cells was standardised by adjusting the absorbance at 250 nm to a value of 0.05 and aliquots of 40 µl were added to each of 30 tubes. Ten microliters of 500 µg/ml rifampicin in dimethylsulphoxide (DMSO) was added to 15 of the tubes and 10 µl of DMSO alone was added to the remaining 15 tubes. All tubes were mixed well and incubated at room temperature for 20 minutes. Fifty microliters of water containing 8.3, 0.2, 0.1 and 0.05 mM of sodium hypochlorite was added to three tubes from the rifampicin-treated group and 3 tubes from the untreated group. Fifty microliters of sterile high-pure water was added to each of 3 tubes from the rifampicin-treated and the untreated groups to serve as no treatment controls. All tubes were incubated at room temperature for 30 minutes and the oxidative dissociation products of NaOCl were neutralised by addition of 30 µl of 7 g/L sodium thiosulphate with mixing. RNA was extracted from all tubes by addition of 500 µl of TRI Reagent using the same procedure described previously. Reverse transcription was also performed as described previously.

#### **4.2.9 Assessing disinfectant efficacy based on DNA and RNA quantification**

Purified *C. burnetii* cells were treated with sodium hypochlorite, hydrogen peroxide and ultraviolet radiation according to the procedures described in Chapter 3. The final concentrations of NaOCl were 0.4, 0.2, 0.1 mM and 4.7 mM (100% kill control), the concentrations of H<sub>2</sub>O<sub>2</sub> were 2, 1 and 0.5% and the doses of UV

radiation delivered were 10, 5 and 1 mJ/cm<sup>2</sup>. Following treatment with the two chemical agents oxidative species were quenched by addition of 30µl of 7g/L sodium thiosulphate with mixing. RNA was extracted, purified and reverse transcribed as described previously and results from these experiments will hereafter be referred to as being generated from quantifying 'cDNA'. In addition, whole genomic DNA was concurrently isolated and purified according to the supplementary protocol described in the technical literature on the Ambion website ([www.ambion.com/techlib/append/supp/tri.pdf](http://www.ambion.com/techlib/append/supp/tri.pdf), accessed on August 12<sup>th</sup>, 2007) and estimates of disinfectant efficacy based upon DNA purified in this way will be referred to as 'TRI DNA' results. The efficacy of each disinfectant was compared to the results generated using the same assays performed in Chapter 3. The results of these assays will be referred to as 'tissue culture'.

#### **4.2.10 TaqMan Quantitative PCR for DNA and cDNA**

DNA extracts were tested by real-time PCR using the protocol described previously for the IS1111a TaqMan primer and probe set. All cDNA samples from the heat response experiment were interrogated using the TaqMan primer/probe oligonucleotide sets ScvA, HspB, 16S and SpolIIE. Conditions for each test can be seen in Chapter 2. All other cDNA samples were assessed using only the ScvA primer and probe set according to the conditions described previously.

Standards were constructed as described previously and were included, in duplicate, in all qPCR runs. No template controls were also included with every run to ensure the absence of contamination.



#### 4.2.11 Data Analysis

The Rotorgene<sup>®</sup> 3000 software was used to automatically select optimal cycle threshold cut-offs based upon the slope of the standard curve and the  $R^2$  value. The user-defined DNA concentration of the standards was then used by the software to provide estimates of the DNA or cDNA concentration of the unknown samples. Where appropriate, Microsoft Excel was used to perform basic calculations and to display results graphically.

The estimated cDNA concentrations at each time point for samples that had been incubated at a particular temperature were normalised by dividing them by the time zero concentration. The statistical significance of any differences observed in the normalised cDNA concentrations at each temperature and time point and the mean cDNA copy number at each time point and effect of were determined by performing a one-way analysis of variance (ANOVA) and Tukey's honestly significant differences test at a 95% confidence limit using the statistical package for social sciences (SPSS version 15.0, SPSS Inc., Chicago, USA).

Quantitative PCR was used to estimate the *C. burnetii* nucleic acid concentration (in copies/genome equivalents per reaction) of a test sample ( $t$ ), each of which was then compared to the nucleic acid concentration of the no-treatment controls (NT) to give a proxy measure of disinfectant efficacy hereafter referred to as 'percentage reduction in DNA/RNA' (R). The equation for calculating this measure can be expressed as:

$$R = (t / NT) \times 100$$

The statistical significance of any differences in the mean Ct value from each treatment group was determined using a one-way analysis of variance (ANOVA)

and post hoc analysis using Tukey's honestly significant differences test at a 95% confidence limit and the independent samples t-test with assumed equal variance was used when comparisons were made between only two data sets.

### **4.3 Results**

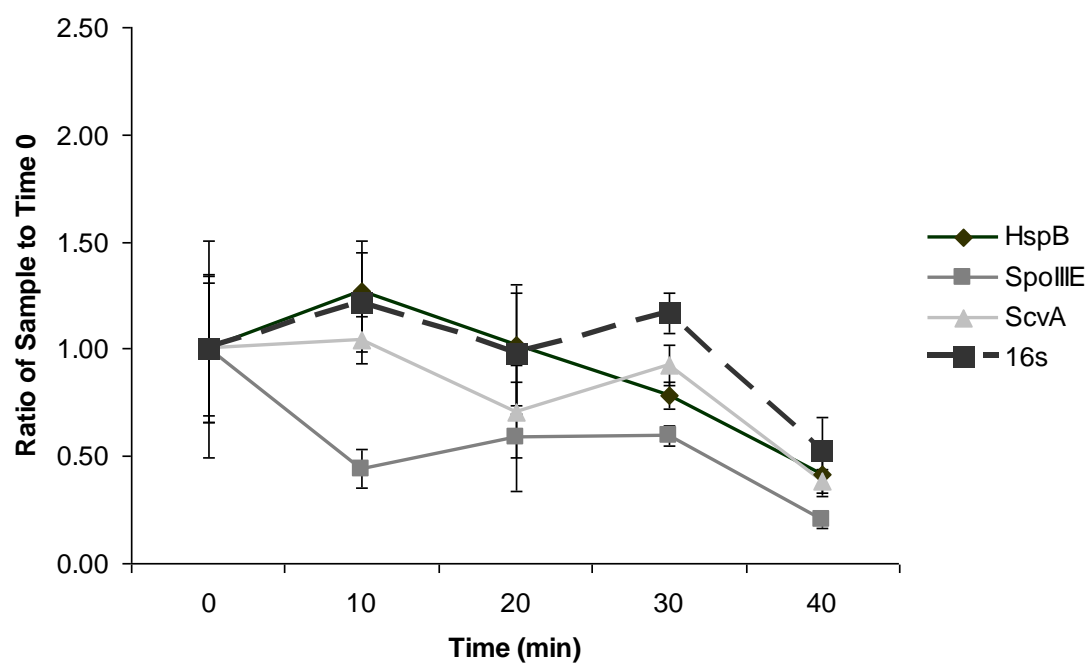
#### **4.3.1 Changes in RNA Transcription in Response to heat**

When the average 16S transcript copy numbers for each temperature, over all time points, were compared it was found that the samples incubated at 40°C had significantly greater relative abundance of mRNA compared to estimates from samples incubated at 37°C and 48°C ( $p < 0.05$ ). There was significantly greater relative abundance of *HspB* transcripts from samples incubated at 40°C compared to samples incubated at 44°C and 48°C ( $p < 0.05$ ). There was a significant decrease in the relative abundance of *icd* and *SpolIII*E transcripts in samples incubated at 37°C compared to samples incubated at 40°C and 48°C ( $p < 0.05$ ). There was a significant increase in the relative abundance of *ScvA* transcripts in samples incubated at 40°C compared to samples incubated at 37°C and 48°C ( $p < 0.005$ ).

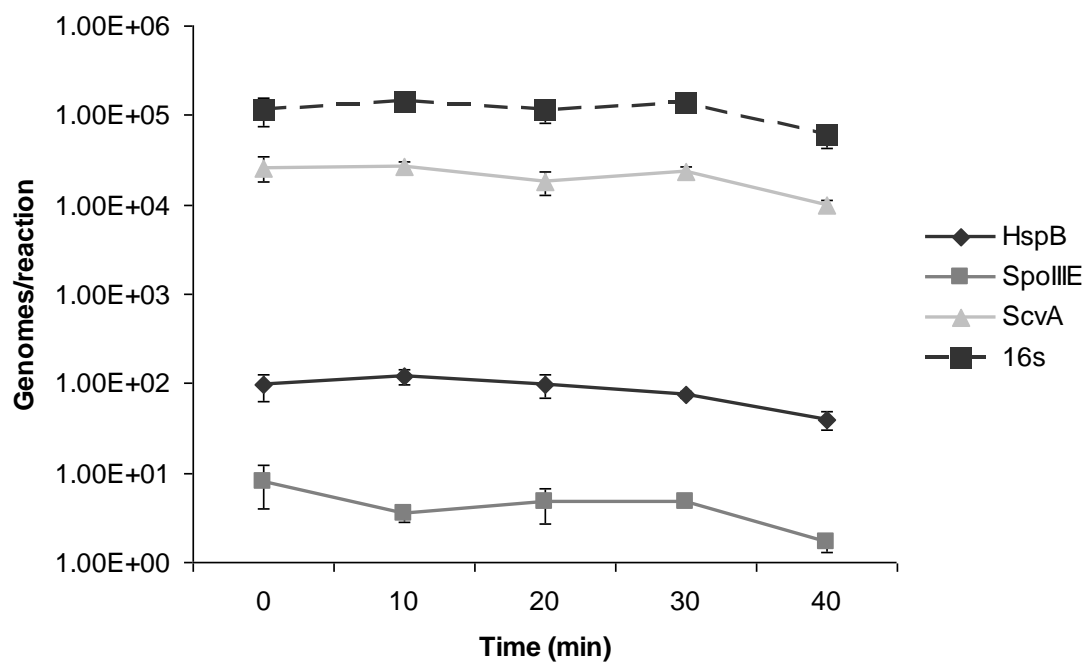
##### **4.3.1.1 Changes in RNA Transcription observed at 37°C**

Changes in the relative abundances of each transcript at 37°C over time and changes in the estimated copy number of each transcript over time at 37°C are shown in Figures 4.1 and 4.2 respectively. There were no significant changes in the normalised abundance of any of the five transcripts over time at 37°C ( $p > 0.05$ ). There were significantly more copies of 16S mRNA compared to the number of copies of each transcript over all ( $p < 0.001$ ). No other significant differences were observed at this temperature.

**Figure 4-1. Changes in the abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at 37°C and expressed as a ratio of the time 0 RNA abundance**



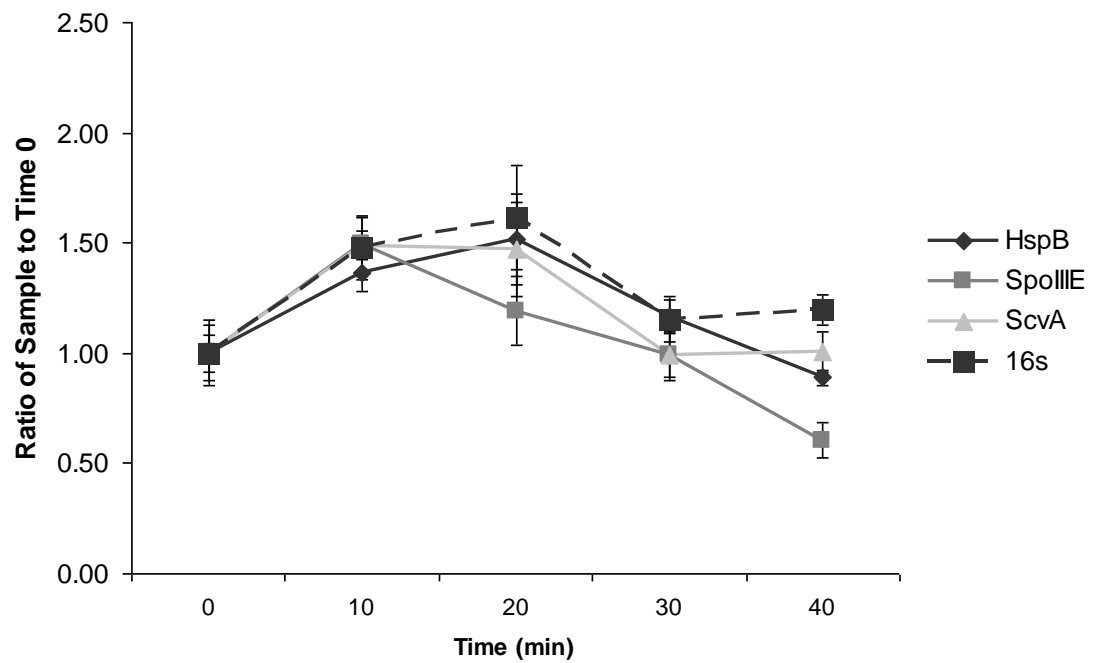
**Figure 4-2. Changes in the absolute abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at over time at 37°C**



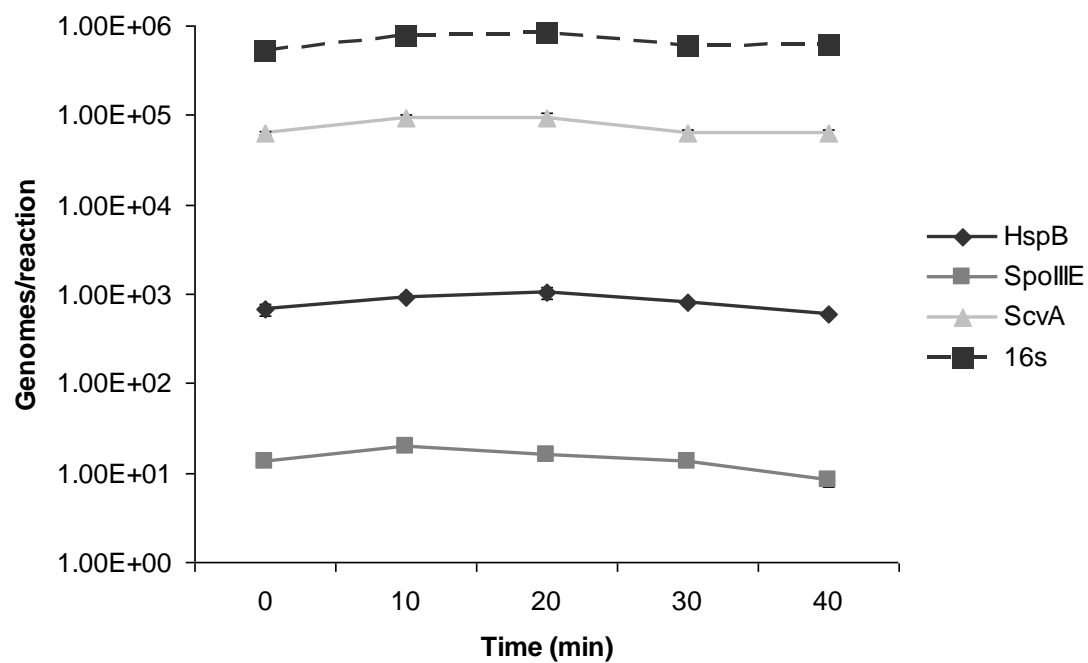
#### 4.3.1.2 Changes in RNA Transcription observed at 40°C

Changes in the normalised abundances of each transcript at 40°C over time and the estimated copy number of each transcript over time at 40°C are shown in Figures 4.3 and 4.4 respectively. The normalised abundance of the *16S* and *HspB* transcripts were significantly increased in samples incubated at 40°C for 20 minutes compared to samples at time zero ( $p<0.05$ ). There was a significantly greater *SpolIII* mRNA population in samples 10 minutes after incubation at 40°C compared to samples incubated for zero, 30 and 40 minutes ( $p<0.05$ ) and in samples incubated for 20 minutes compared to samples incubated for 40 minutes ( $p<0.005$ ). The mean abundance of *16S* mRNA was significantly greater compared to all other transcripts at all time points ( $p<0.001$ ). The *ScvA* concentration was significantly greater compared to all transcripts other than *16S* ( $p<0.01$ ).

**Figure 4-3.** Changes in the abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at 40°C and expressed as a ratio of the time 0 RNA abundance



**Figure 4-4. Changes in the absolute abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at over time at 40°C**

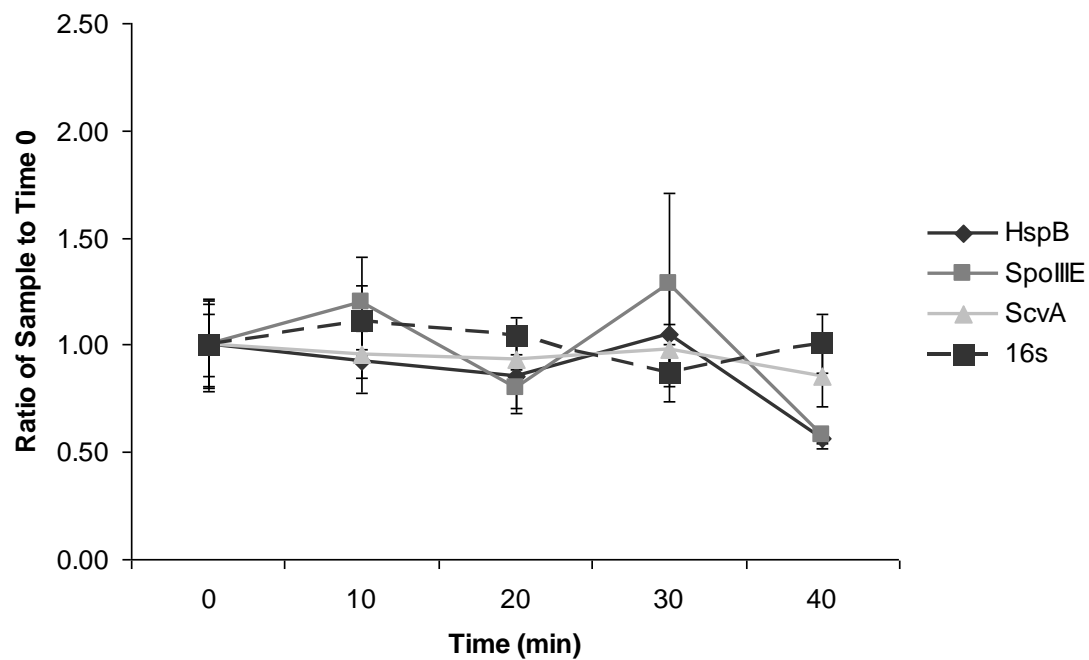


#### **4.3.1.3 Changes in RNA Transcription observed at 44°C**

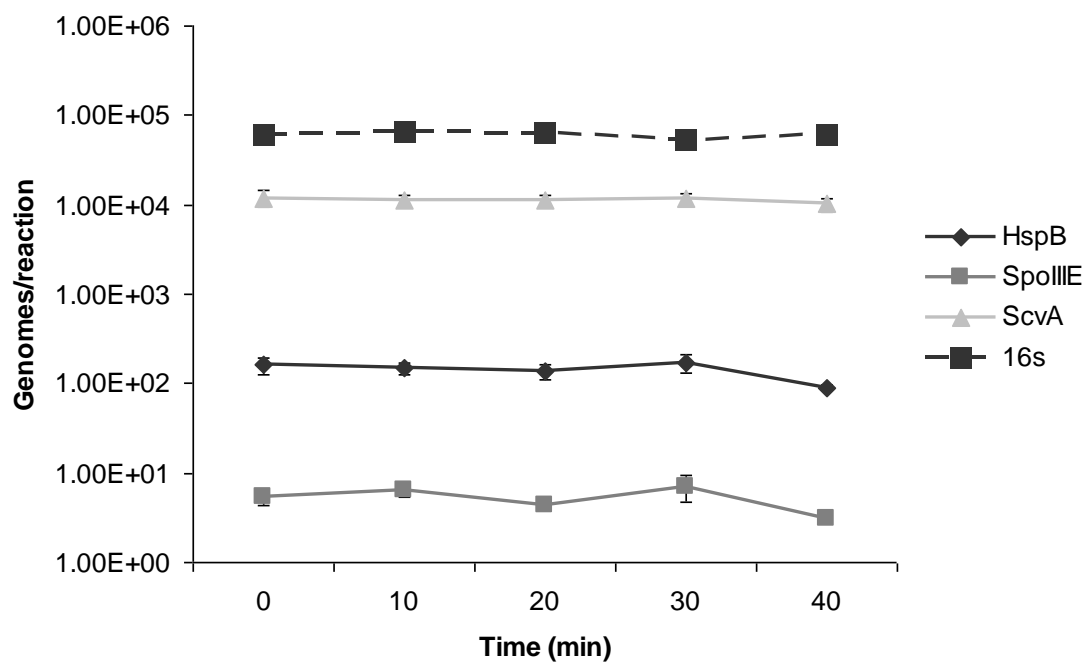
Changes in the relative abundances of each transcript in samples incubated at 44°C over time and the estimated copy number of each transcript in samples over time are shown in Figures 4.5 and 4.6 respectively. There were no significant changes in the normalised abundance of any of the five transcripts at each time point ( $p>0.05$ ). The number of copies of the 16S and ScvA mRNA were significantly greater than all other transcripts ( $p<0.001$ ). There was no significant difference between the number of copies of 16S and ScvA mRNA ( $p<0.001$ ).



**Figure 4-5.** Changes in the abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at 44°C and expressed as a ratio of the time 0 RNA abundance



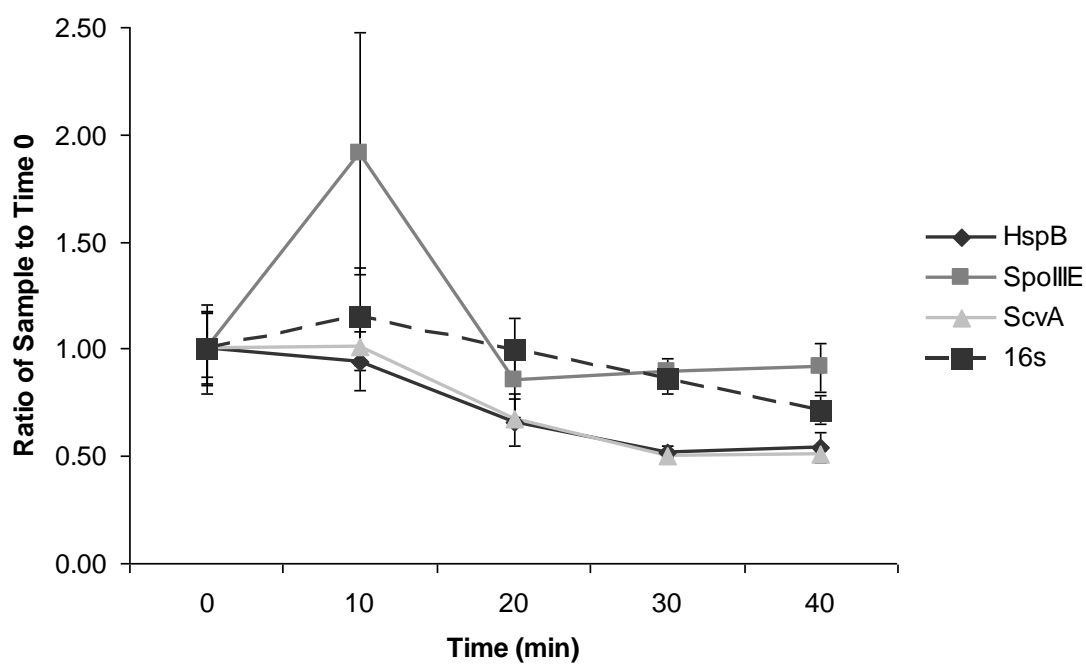
**Figure 4-6. Changes in the absolute abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at over time at 44°C**



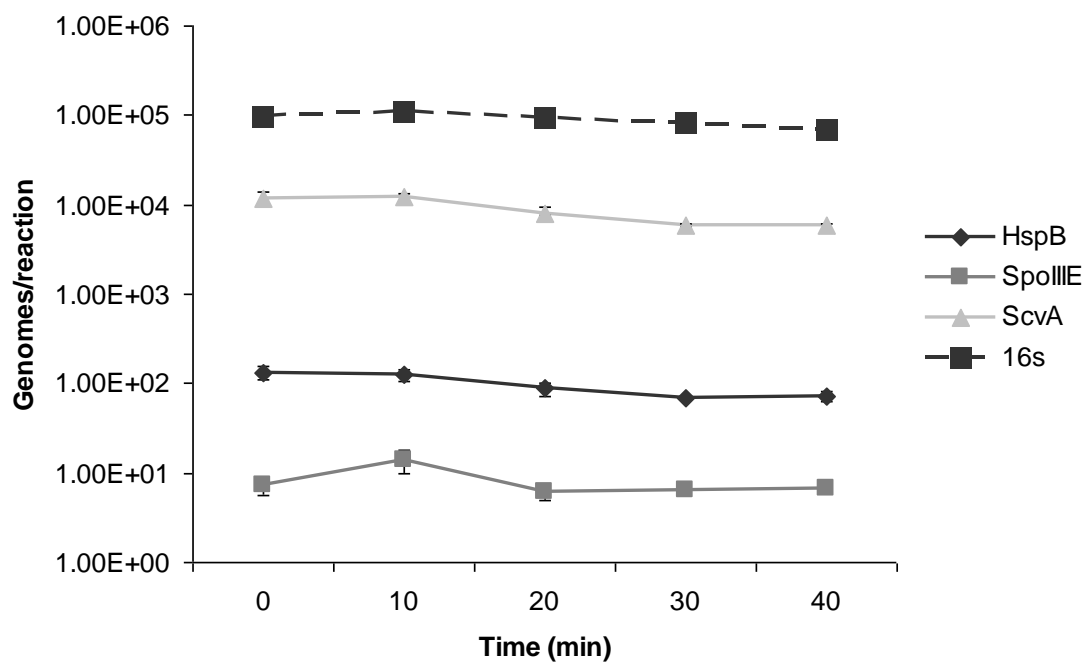
#### 4.3.1.4 Changes in RNA Transcription observed at 48°C

Changes in the normalised abundances of each transcript at 48°C over time and the estimated copy number of each transcript over time at 48°C are shown in Figures 4.7 and 4.8 respectively. The normalised abundance of the *HspB* transcript was significantly decreased in samples incubated at 48°C for 30 minutes compared to samples at time zero ( $p<0.05$ ). There was a significant decrease in the abundance of the *ScvA* transcript in samples incubated at 48°C for 30 and 40 minutes compared to samples incubated for zero and 10 minutes ( $p<0.05$ ). There were significantly more copies of 16S mRNA compared to each other transcript at each time point ( $p<0.001$ ).

**Figure 4-7. Changes in the abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at 48°C and expressed as a ratio of the time 0 RNA abundance**



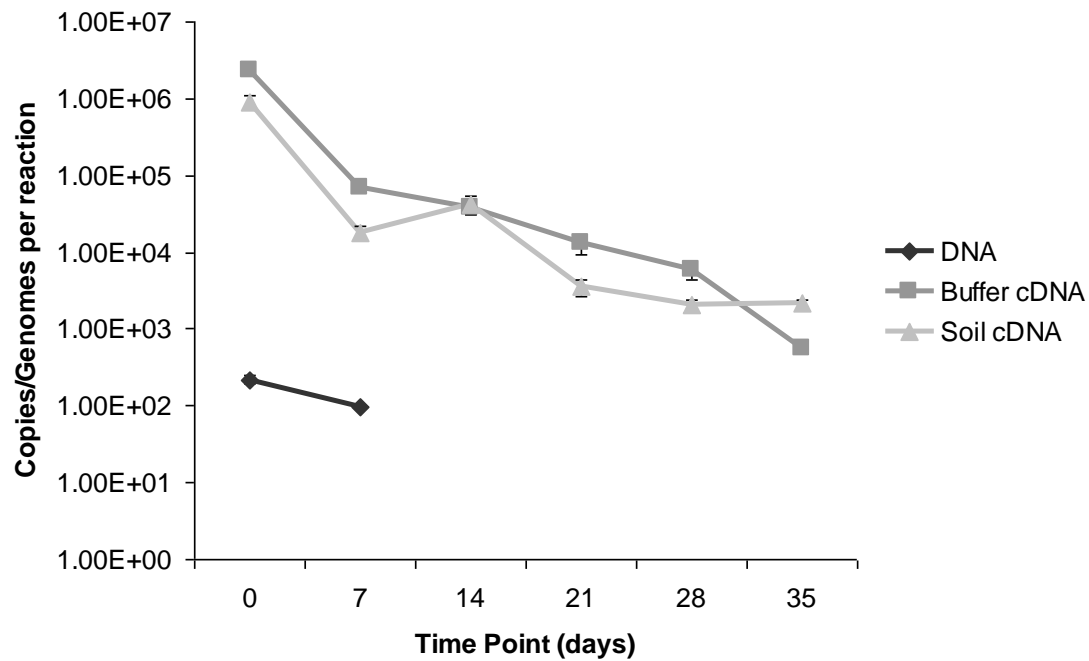
**Figure 4-8. Changes in the absolute abundance of five *Coxiella burnetii* RNA transcripts estimated by qPCR over time at over time at 48°C**



#### **4.3.2 RNA and DNA extracted from soil over time**

Figure 4.9 shows the amount of DNA and cDNA that was detectable by qPCR in soil over time. The amount of cDNA that was detectable in buffer alone was also plotted for comparison. No detectable naked DNA was present in the soil sample after day seven while cDNA was present in both soil and buffer at the last time point (35 days) of the experiment.

**Figure 4-9. Copy number of *Coxiella burnetii* cDNA and DNA purified from soil or water over time estimated by qPCR**

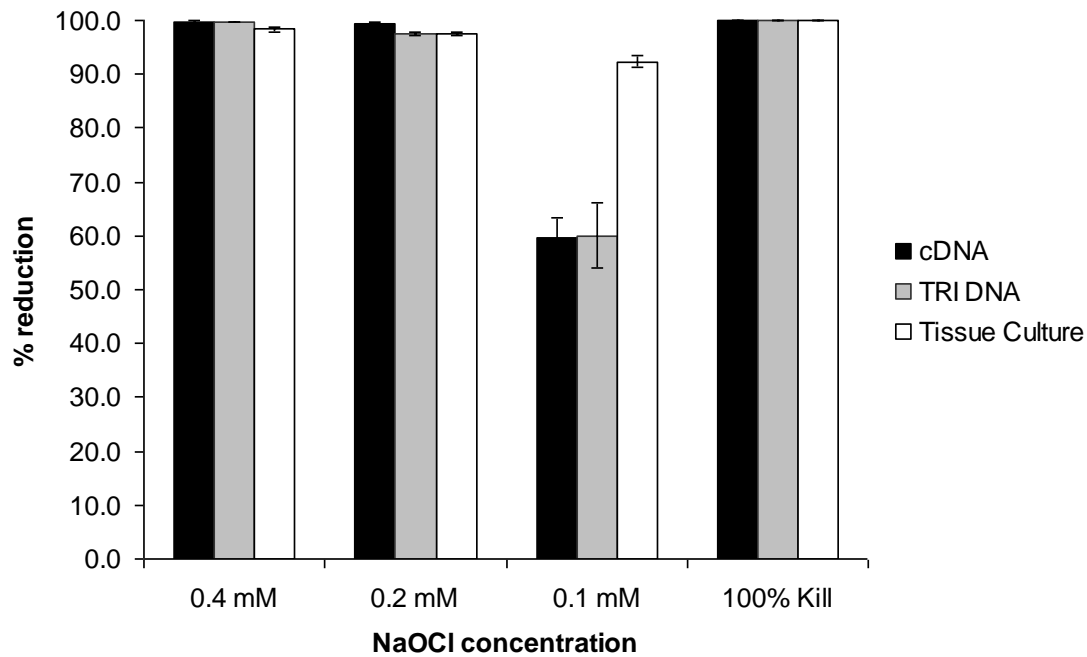


#### **4.4 Assessment of Disinfectant Efficacy of Sodium Hypochlorite**

The percentage reductions in DNA/RNA from cultures using *C. burnetii* exposed to 0.4, 0.2, 0.1 mM sodium hypochlorite and 100% kill estimated using qPCR of cDNA, TRI extracted DNA and DNA extracted from tissue culture are displayed in Figure 4.10.



**Figure 4-10. Comparison of measurements of NaOCl disinfectant efficacy using qPCR of 'cDNA', 'TRI DNA' and DNA purified from *C. burnetii* after culture for seven days *in vitro***



#### **4.4.1 Differences between disinfection efficacy of varying NaOCl concentrations**

##### **4.4.1.1 Quantified with qPCR of cDNA**

There was a significant decrease in the number of *C. burnetii* cDNA transcripts detected in samples that were exposed to each NaOCl concentration and the 100% kill control compared to the NT control ( $p<0.05$ ). There were significantly more *C. burnetii* cDNA transcripts detected in samples exposed to 0.1 mM NaOCl compared to the 100% kill control, 0.2 mM NaOCl and 0.4 mM NaOCl ( $p<0.05$ ). There was no significant difference in the genome equivalents detected in samples of *C. burnetii* exposed to 0.2 mM, 0.4 mM NaOCl and the 100% kill control ( $p>0.05$ ).

##### **4.4.1.2 Quantified with qPCR of DNA co-purified during RNA extraction**

There was a significantly lower concentration of *C. burnetii* DNA in samples exposed to all NaOCl concentrations and the 100% kill control compared to the NT control ( $p<0.001$ ). There was a significantly higher DNA concentration in samples exposed to 0.1 mM NaOCl compared to the 100% kill control and significantly less DNA compared to the samples exposed to 0.2 mM and 0.4 mM NaOCl ( $p<0.05$ ). There were no significant differences in the estimated number of genome equivalents in the 0.2 mM and 0.4 mM NaOCl treatments or the 100% kill control ( $p>0.05$ ).

#### **4.4.1.3 Quantified with qPCR of DNA extracted from *Coxiella burnetii* after *in vitro* culture for seven days**

There were significantly less *C. burnetii* genome equivalents detected in samples that were exposed to each NaOCl concentration and the 100% kill control compared to the NT control ( $p<0.05$ ). The samples treated with 0.1 mM NaOCl were found to contain significantly more *C. burnetii* genome equivalents than the 100% kill control ( $p<0.01$ ).

#### **4.4.2 Differences between measurement of percentage reduction of cDNA, DNA co-purified with RNA and DNA extracted from tissue culture**

The estimated reduction in the number of *C. burnetii* genome equivalents in samples exposed to 0.1 mM NaOCl was significantly lower when data from the tissue culture method was used compared to data from the cDNA or TRI DNA methods ( $p<0.05$ ).

The estimated reduction in the number of *C. burnetii* genome equivalents in samples exposed to 0.2 mM NaOCl was significantly greater when data from the cDNA method was used compared to data from the tissue culture method or the TRI DNA method ( $p<0.001$ ).

The estimated reduction in the number of *C. burnetii* genome equivalents in samples exposed to 0.4 mM NaOCl was significantly greater when data from the tissue culture method was used compared to data from the cDNA and TRI DNA methods ( $p<0.001$ ).

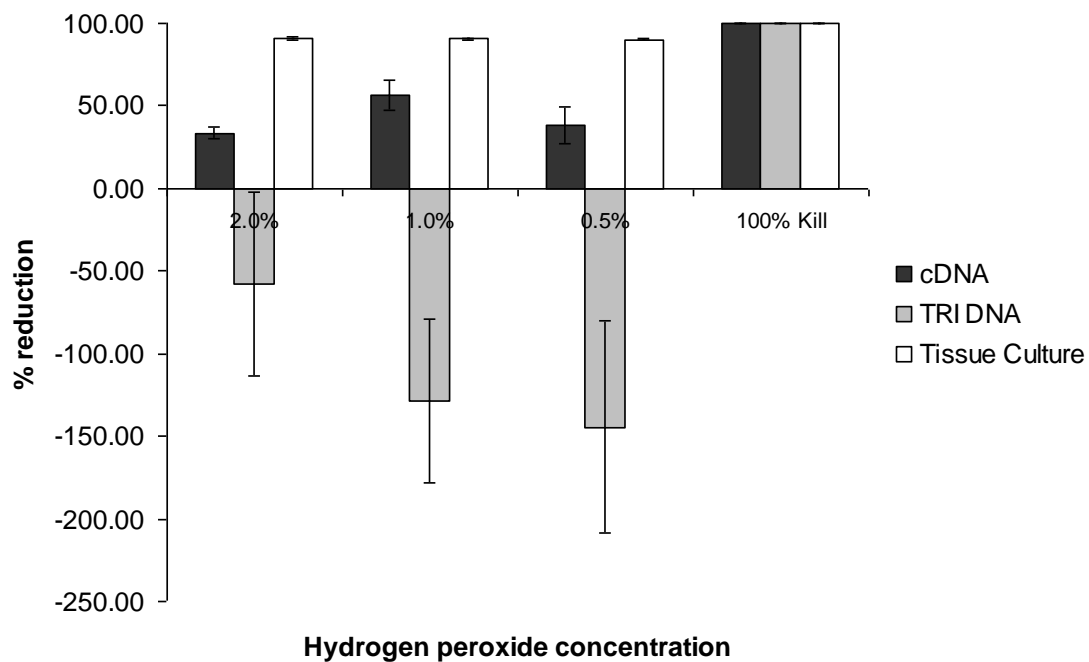
The estimated reduction in *C. burnetii* transcript numbers in samples exposed to the 100% kill control was significantly greater than the reduction quantified when

data from the tissue culture method was used compared to data from the TRI DNA method ( $p<0.005$ ).

#### **4.5 Assessment of Disinfectant Efficacy of Hydrogen Peroxide**

The percentage reductions in DNA/RNA from cultures using *C. burnetii* exposed to hydrogen peroxide and the 100% kill control estimated using qPCR of cDNA, TRI extracted DNA and DNA extracted from tissue culture are displayed in Figure 4.11.

**Figure 4-11. Comparison of measurements of hydrogen peroxide disinfectant efficacy using qPCR of 'cDNA', 'TRI DNA' and DNA purified from *Coxiella burnetii* after *in vitro* culture for seven days**



#### **4.5.1 Differences between disinfection efficacy of varying H<sub>2</sub>O<sub>2</sub> concentrations**

##### **4.5.1.1 Quantified with qPCR of cDNA**

There was a significant decrease in the number of *C. burnetii* cDNA transcripts detected in samples that were exposed to each H<sub>2</sub>O<sub>2</sub> concentration and the 100% kill control compared to the NT control ( $p<0.001$ ). There were significantly less *C. burnetii* cDNA genome equivalents detected in samples exposed to 0.5 mM H<sub>2</sub>O<sub>2</sub> compared to samples exposed to the 100% kill control and 1% H<sub>2</sub>O<sub>2</sub> but significantly more genome equivalents than was estimated for samples exposed to 2% H<sub>2</sub>O<sub>2</sub> ( $p<0.005$ ). The estimated genome equivalents in samples exposed to 1 % and 2% H<sub>2</sub>O<sub>2</sub> were not significantly different from each other or from samples exposed to the 100% kill control.

##### **4.5.1.2 Quantified with qPCR of DNA co-purified during RNA extraction**

There was a significantly lower concentration *C. burnetii* DNA in samples exposed to all concentrations of H<sub>2</sub>O<sub>2</sub> and samples exposed to the 100% kill control compared to the NT control ( $p<0.001$ ). There was a significantly higher DNA concentration in samples exposed to 0.5 % H<sub>2</sub>O<sub>2</sub> compared to samples exposed to the 100% kill control, 1% and 2% H<sub>2</sub>O<sub>2</sub> ( $p<0.05$ ). There were no significant differences in the estimated number of genome equivalents in samples exposed to 1 % and 2 % H<sub>2</sub>O<sub>2</sub> treatments compared to samples exposed to the 100% kill control ( $p>0.05$ ).

#### **4.5.1.3 Quantified with qPCR of DNA extracted from *Coxiella burnetii* after *in vitro* culture for seven days**

There was significantly more *C. burnetii* DNA in samples exposed to all concentrations of H<sub>2</sub>O<sub>2</sub> and the 100% kill control compared to the NT control samples ( $p<0.001$ ).

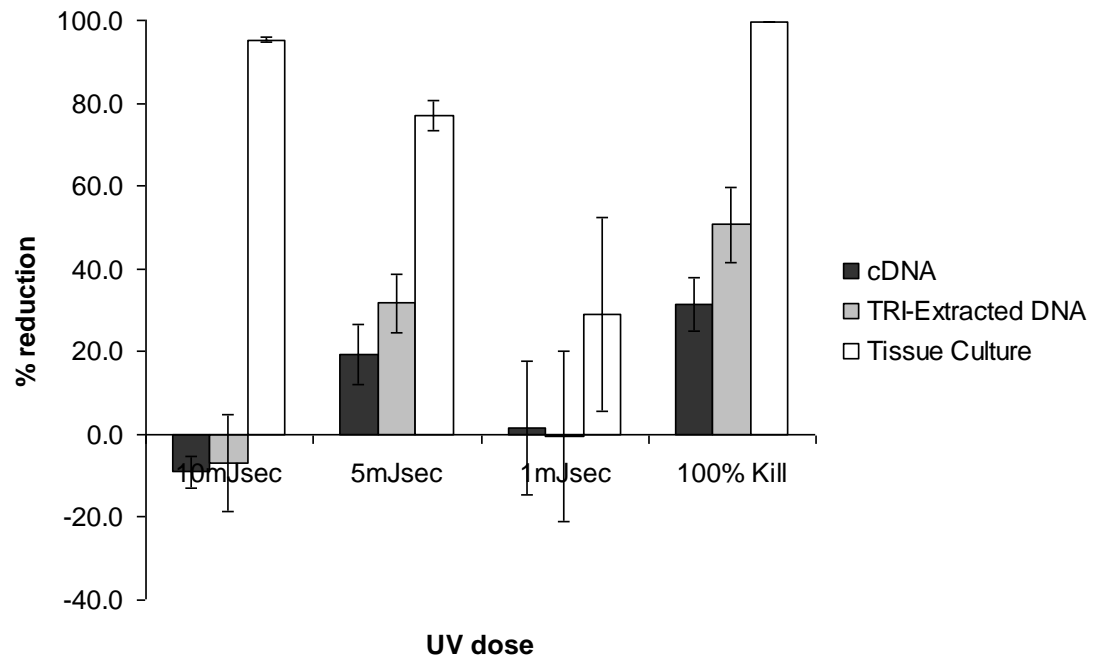
#### **4.5.2 Differences between measurement of percentage reduction of cDNA, DNA co-purified with RNA and DNA extracted from tissue culture**

The estimated number of *C. burnetii* genome equivalents in samples exposed to all concentrations of H<sub>2</sub>O<sub>2</sub> and the 100% kill control were significantly lower when data from the tissue culture method was used compared to data using the cDNA and TRI DNA methods ( $p<0.05$ ).

### **4.6 Assessment of Disinfectant Efficacy of Ultraviolet Light**

The percentage reductions in DNA/RNA resulting from treatment with UV light as estimated using qPCR of cDNA, TRI extracted DNA and DNA extracted from tissue culture are displayed in figure 4.12.

**Figure 4-12. Comparison of measurements of UV radiation disinfectant efficacy using qPCR of 'cDNA', 'TRI DNA' and DNA purified from *Coxiella burnetii* after *in vitro* culture for seven days**





#### **4.6.1 Differences between disinfection efficacy of varying UV doses**

##### **4.6.1.1 Quantified with qPCR of cDNA**

No significant differences were observed between any treatments or controls using this measurement method.

##### **4.6.1.2 Quantified with qPCR of DNA co-purified during RNA extraction**

Samples exposed to 10 mJsec<sup>-1</sup> of UV radiation had a significantly lower DNA concentration compared to samples exposed to the 100% kill control ( $p<0.05$ ). No other significant differences were observed.

##### **4.6.1.3 Quantified with qPCR of DNA extracted from *Coxiella burnetii* after *in vitro* culture for seven days**

There was a significantly greater reduction in *C. burnetii* genome equivalents in samples exposed to all UV treatment doses, except 1 mJsec<sup>-1</sup>, and the 100% kill control compared to the NT control ( $p<0.005$ ). Samples exposed to 10 mJsec<sup>-1</sup> had significantly more *C. burnetii* genome equivalents compared to samples exposed to 1 mJsec<sup>-1</sup> ( $p<0.05$ ).

##### **4.6.1.4 Statistical differences between measurement of percentage reduction of cDNA, DNA co-purified with RNA and DNA extracted from tissue culture**

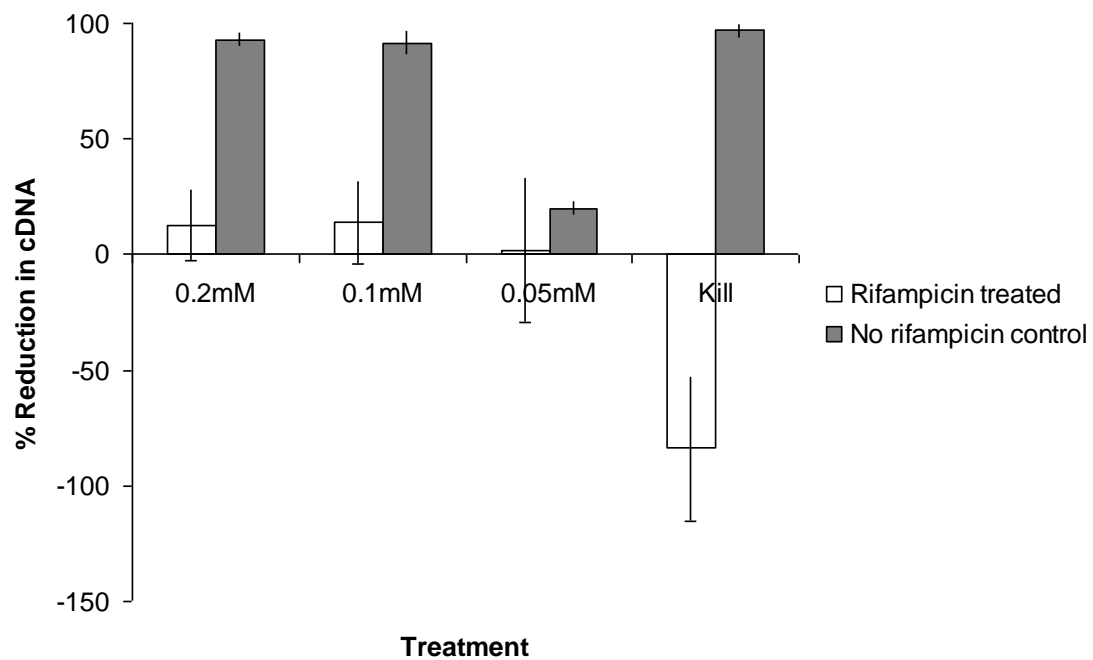
The estimated reduction in *C. burnetii* genome equivalents observed for samples treated with 10 mJsec<sup>-1</sup>, 5 mJsec<sup>-1</sup> UV and the 100% kill control using data from the tissue culture method were significantly greater than the reduction quantified using the cDNA and TRI DNA methods ( $p<0.001$ ). No significant differences were observed in the estimated reduction in genome equivalents

between the three methods when the *C. burnetii* cells were treated with 1 mJsec<sup>-1</sup> UV radiation and in the NT controls.

#### **4.7 Blocking RNA Transcription with Rifampicin**

The reductions in *C. burnetii* RNA in cultures using *C. burnetii* treated with rifampicin prior to exposure with NaOCl and *C. burnetii* which had not been pre-treated with rifampicin are shown in figure 4.13.

**Figure 4-13. Quantitative PCR measurement of *Coxiella burnetii* cDNA reduction in comparison to a no treatment control in response to insult with NaOCl where one group of samples was pre-treated with rifampicin**



#### **4.7.1 Differences between samples pre-treated with rifampicin and samples not pre-treated with rifampicin**

The estimated reduction in RNA was significantly less in cultures using *C. burnetii* that had been pre-treated with rifampicin and exposed to 0.2 mM NaOCl, 0.1 mM NaOCl and the kill controls compared to cells that had not been pre-treated ( $p < 0.005$ ). There were no significant differences in the estimated reduction in RNA for the no treatment controls or the 0.05 mM NaOCl-treated samples.

#### **4.7.2 Differences between disinfection efficacy of varying NaOCl concentrations where all samples were pre-treated with rifampicin**

There was significantly greater reduction of RNA in all samples that were pre-treated with rifampicin and exposed to 0.2 and 0.1 mM NaOCl compared to the 100% kill control ( $p < 0.05$ ).

### **4.8 Discussion**

#### **4.8.1 Transcription of *Coxiella burnetii* genes in response to heat**

The most dramatic and sustained increase in *C. burnetii* transcriptional activity was observed at a temperature of 40°C where cDNA quantity remained at, or above, the time zero mark for all RT-PCR targets examined. However, the greatest post-heat treatment increase in RNA quantity was observed from the *SpolIIE* and *icd* genes when *C. burnetii* was exposed to 48°C for 10 minutes. At this time point the amount of RNA was almost double that found in the time zero controls indicating a substantial increase in the transcription of these two genes. It is interesting to note that the relative transcription of *HspB* was at, or below, a sample to time zero control ratio of 1.05 for all time points at 44°C and 48°C. It

was anticipated that transcription of heat shock genes would increase in response to elevated temperature so this result was unexpected. This trend may indicate that heat resistance of the SCV of *C. burnetii* is due to the increased peptidoglycan content of its cell wall in comparison to the LCV (Amano, Williams et al. 1984) rather than an active transcriptional response to heat stress. Additionally, the condensed, protein-stabilised state of the SCV chromatin (Nermut, Schramek et al. 1968) may provide protection for the organisms genetic material, again precluding the need for an active response to a heat-challenge.

Inducing an increase in transcriptional activity is useful for definitively identifying viable cells and increasing the chance of detecting them. However, an assay of this nature is limited by the abundance of each RNA species. For this reason the total copy number of each transcript present in the cell is also important for the development of a sensitive viability assay.

For all temperatures and time points the number of 16S RNA transcripts was 5 to 10-fold greater than any other RNA tested for. However, the RT-qPCR targeting cDNA from this RNA was found to have unacceptably poor specificity (see Chapter 2) for an assay that would be applied to environmental samples containing mixed microbial communities. Furthermore, ribosomal RNA is stabilised by proteins which may enable it to persist after the death of the cell (Jenkins, Trout et al. 2003) and this could confound a viability assay targeting this type of RNA.

The next most abundant transcript was produced from the *ScvA* gene which in turn was 50 to 300 fold more abundant than any of the other transcripts at all time points and temperatures tested. The PCR targeting the reverse transcribed RNA for the *ScvA* gene was also highly specific and theoretically capable of detecting

as few as 1.6 transcripts per reaction, although at this concentration only one out of every six reactions produced detectable amplification (see Chapter 2). The protein encoded by the *ScvA* gene is believed to be involved in the condensation of chromatin (Heinzen and Hackstadt 1996) and is most transcriptionally active at the onset of the lifecycle phase of *C. burnetii* that has been likened to the stationary phase of other bacteria (Coleman, Fischer et al. 2004). However, it is not known how long this RNA remains detectable in the cell.

#### **4.8.2 Purification and quantification of *Coxiella burnetii* *ScvA* RNA from soil over time**

To determine if a RT-qPCR targeting the *ScvA* transcript was useful for the detection of viable *C. burnetii* cells in environmental samples a longitudinal experiment that involved extracting RNA from soil containing *Coxiella* cells was performed at seven day intervals. The finding that RNA from both the buffer and soil samples was still detectable for the entire length of the experiment (35 days) despite the absence of naked DNA after the first 7 days was important. The observation that RNA decreased over time may be attributable to cell death and subsequent degradation or down regulation of transcriptional activity. If the former is true it must be considered that, in this instance, phase II *C. burnetii* may not be an ideal model for wild-type cells. Lipopolysaccharide contributes to the intrinsic resistance exhibited by Gram-negative bacteria to environmental stressors (McDonnell and Russell 1999) but the truncated LPS of phase II *C. burnetii* cells may not afford the same level of protection. Therefore, if the decrease in RNA concentration over time observed in this experiment was due to cell death rather than metabolic dormancy phase I cells in environmental matrices may well be detectable for a longer duration. Conversely, if the downward trend in RNA quantity is due to reduction in transcriptional activity but not cell death the results

would indicate that the method is not applicable indefinitely but has a useable range of at least 35 days. This interpretation is supported by the results of work conducted with *C. parvum* that showed that while cells were undetectable by RT-qPCR after three months storage they were still infectious according to cell culture-PCR and mouse inoculation tests (Jenkins, Trout et al. 2003).

#### **4.8.3 Comparison of methods to evaluate *Coxiella burnetii* viability**

To challenge the above interpretation of the data from this study a series of experiments was conducted to compare the RT-qPCR described here to the cell culture-PCR described in Chapter 3. For the purposes of these experiments a proxy measure was used to estimate *C. burnetii* viability. By comparing the amount of *C. burnetii* DNA in a disinfectant-treated sample with the amount of *C. burnetii* DNA in an untreated control the ability of the coxiellae to infect and divide within the host cells could be measured. This measure was referred to as 'percentage reduction in DNA/RNA' but the comparison between treated samples and untreated controls can more accurately be seen as an indirect measure of infectivity and replicative competence. Similarly, comparison of the abundance of a particular mRNA species in treated samples with the abundance in untreated controls can give a measure of the effect that a disinfectant has on the transcriptional ability of *C. burnetii*. Transcriptional activity is considered to be a measure of cellular viability in many instances (Keer and Birch 2003) and thus here 'percentage reduction in RNA' can be used to quantify disinfectant efficacy. For both DNA and RNA a 'reduction' of 100% indicates that the treated sample contains an amount of DNA or RNA that is less than 0.01% of the amount measured in the untreated controls. Overall the results showed that there was good agreement between the level of cDNA and DNA co-purified during the procedure to extract RNA from *C. burnetii* cells exposed to NaOCl, hydrogen

peroxide and UV radiation. This observation is not surprising given that the reduction caused by the treatments probably resulted in dramatic damage to the cell wall and therefore unregulated traffic between the cell interior and the external environment. Therefore, RNA and DNA are likely to be degraded in the same manner. This is supported by the results from the experiment performed with UV radiation where, with one exception, none of the treatments or controls were significantly different from each other or the controls when measured using cDNA or DNA co-purified during the RNA extraction. Exposure to UV radiation causes the formation of thiamidine dimers in DNA which makes it impossible for the affected cell to divide or gain access to single stranded nucleotides for transcription of RNA (Shin, Linden et al. 2001). Ultra violet light-treated cells retain metabolic function, which was evidenced by the lack of difference in the amount of RNA or DNA that was detected immediately after treatment. In contrast, the *in vitro* culture assay showed that UV radiation inhibited the replicative ability of the cells. These observations serve to highlight that a viability assay based upon relative RNA abundance can only be used to measure agents that are bactericidal in nature, not bacteristatic. However, while measurement of viability using RNA was, in most instances, different from the measurement provided by the *in vitro* cell culture-PCR assay, significant differences were observed between treatment concentrations and controls using the RT-qPCR method. This indicates that the assay has the dynamic capacity to discriminate between varying levels of viability for a given number of cells.

#### **4.8.4 The effect of blocking *Coxiella burnetii* transcription with rifampicin on the RT-qPCR viability assay**

The transcripts from housekeeping genes appear to be the most promising targets for a viability assay for *C. burnetii* because transcription of these genes,



while not completely stable between tissue types, occurs at a relatively stable rate irrespective of any external stimuli (Vandesompele, De Preter et al. 2002). However, assays targeting these highly conserved oligonucleotides can lack specificity as seen in the results of this study. Choosing a more unique gene such as *ScvA* can produce a more specific test but the transcript may not be produced in detectable numbers at all times thus potentially decreasing the sensitivity of the test. The results of this study show that the *ScvA* transcript can be used as a proxy measure of viability but it was not clear whether the amplified RNA was from a static pool or was constantly being degraded and then synthesised *de novo* in response to the external stimuli applied. Results from attempts to block transcription with rifampicin showed that treating the *C. burnetii* cells with this antibiotic prior to exposure to chemical insult appears to arrest the synthesis of new RNA. This result was expected because rifampicin inactivates RNA polymerase (Stryer 1995). Furthermore, degradation of RNA does not seem to occur following treatment with this antibiotic. It is possible that the time frame between the addition of rifampicin and the RNA extraction solution was insufficient for RNases and passive RNA decay to break down the transcripts present in the cells (Deutscher 2006) or perhaps that while the concentration of DMSO used to dissolve the rifampicin was sub-inhibitory to cell growth (Karlson and Ulrich 1969) it may have had a deleterious effect on enzymes normally involved in RNA degradation (Deutscher 2006). It is likely that the reduction in RNA abundance seen in samples not exposed to rifampicin and subsequently exposed to NaOCl is due to active degradation by the cells. This response may be a result of the cell reallocating resources to mobilise a defence against the chemical stressor. This response may be involved with altering the permeability of the membrane to exclude the chemical or perhaps the up regulation of repair mechanisms for oxidative damage (Mertens, Lantsheer et al. 2005). Alternatively, the reduced

RNA quantity may have been due to damage of the RNA itself and of the enzymes involved in *de novo* synthesis of new RNA (McKenna and Davies 1988). It is more difficult to explain the observation that RNA abundance was increased in cells that were pre-treated with rifampicin and exposed to the '100% kill control' concentration of NaOCl (4.17 mM). This response was not observed in the previous NaOCl experiment so it is most likely an anomaly caused by an unequal distribution of *C. burnetii* cells into tubes prior to treatment.

#### 4.8.5 Conclusions

The results from this study show that an RT-qPCR-based approach to prove viability in *C. burnetii* is appropriate in most circumstances. However, certain improvements could be made to the method described here because reverse transcription of RNA utilises random primer technology which, while resulting in high yields, is thought to reduce assay specificity (Bustin and Nolan 2004). This may also impact on assay sensitivity due to its selective amplification of high-abundance RNA's such as rRNA (Bustin and Nolan 2004). Thus, it may now be beneficial to design sequence-specific reverse transcriptase primers to improve the sensitivity of the assay. It may also be prudent to verify the results presented here with phase I wild type *C. burnetii*. However, there are limited facilities with a sufficient level of biological safety classification to perform these experiments. Some inherent characteristics of the reverse transcription process must also be taken into account when assessing the potential applications for an assay such as is described here. The reverse transcriptase enzyme itself has been shown to be inhibitory to PCR (Suslov and Steindler 2005) and high-abundance transcripts are preferentially reverse transcribed to the exclusion of low-abundance transcripts (Bustin and Nolan 2004). The implications of these findings are that a potential mRNA viability marker must be carefully chosen to ensure that it is not excluded

from the RT process and that the conditions of the RT reactions must be maintained within strict limits to enable quantitative comparisons between samples.

Furthermore, as mentioned in Chapter 3, validation of these results against a mouse inoculation model may be required before the technique can be recognised as a valid approach for determining viability of this environmental pathogen. Even without validation against a mouse inoculation model with phase I cells the assay presented here may prove to be a useful tool for application in livestock-associated industries. Often real-life industrial applications work on risk-reduction strategies as absolute disinfection is not realistic. The RT-qPCR described could be used to provide quantifiable information with defined limitations in regard to detecting viable *C. burnetii* in environmental matrices such as soil.

## **5. An indirect enzyme-linked immunosorbent assay for the detection of anti-*Coxiella burnetii* antibodies in kangaroos**

### **5.1 Introduction**

It has been demonstrated that *C. burnetii* has a strong association with domestic ruminants (Raoult and Marrie 1995) as well as native Australian marsupials (Derrick 1939; Pope, Scott et al. 1960). Thus, the causative agent of Q fever is recognised as a serious occupational hazard for people who work with or around waste and birth products of livestock or marsupials which can harbour infective *C. burnetii* cells (Garner, Longbottom et al. 1997). Despite this there have been no recent studies into the sylvatic reservoirs for *C. burnetii* in Australia. Previous studies used either complement fixation (Dane and Beech 1955; Pope, Scott et al. 1960; Munday 1972) or agglutination tests (Derrick 1939) to detect anti-*C. burnetii* antibodies in marsupial sera. These two tests have advantages over more specific assays such as ELISA because they do not require specific anti-species antibodies and therefore do not require samples from confirmed infected and uninfected animals to serve as controls. However, these assays have relatively poor sensitivity and specificity in comparison with ELISA (Peter, Dupuis et al. 1987; Peter, Dupuis et al. 1988; Fournier, Marrie et al. 1998; Field, Mitchell et al. 2000). A competitive ELISA has been used for detecting the exposure of herds of camels to *C. burnetii* (Soliman, Botros et al. 1992) but competitive ELISA's require monoclonal antibodies (Nielsen 2002) and can be time consuming to develop (Crowther 2000). While the current reference method for detection of Q fever is the immunofluorescence assay (Field, Mitchell et al. 2000) it has been criticised on two points; it has a subjective component and it is not easily standardised (Peter, Dupuis et al. 1988; Field, Mitchell et al. 2000). It is therefore

desirable to develop an indirect ELISA that is compatible with marsupial sera in general, but more specifically with kangaroo sera.

## **5.2 Materials and methods**

### **5.2.1 Animal serum**

Serum and faeces were collected from western grey kangaroos (*M. fuliginosus*) commercially harvested in Western Australia and stored as described in Chapter 6. A “positive” control bovine serum sample was obtained from the CHECKiT ELISA (IDEXX Laboratories Inc., Switzerland). Samples from western barred bandicoots, *Perameles bougainville*, were collected from Dorre Island (25°03’S, 113°06’E) and Bernier Island (24°50’S; 113°08’E) in Western Australia.

### **5.2.2 Development of an enzyme-linked immunosorbent assay to detect infection with *Coxiella burnetii* in kangaroos**

Serum from kangaroos with known exposure status to *C. burnetii* were not available so the development of this test was performed in two stages. In the first stage the compatibility of the reagents was established and a selection of kangaroo test sera was screened to identify high reacting samples. This stage was completed using the ruminant “positive” and “negative” control sera included in the CHECKiT ELISA and Protein G-HRP (Sigma-Aldrich, Saint Louis, Missouri, USA) as a secondary detection antibody. Protein G-HRP binds to ruminant antibodies and also binds to rabbit antibodies. Therefore, an assay utilising rabbit anti-kangaroo antiserum and Protein G-HRP can be used to test ruminant and kangaroo sera on the same plate with minimal modifications. This enabled initial screening of kangaroo sera that had high and low OD values (termed high and low “reactors”). The high and low reactors were then used in the second stage of

development to optimise the assay conditions and evaluate other aspects of the test such as repeatability and compatibility with bandicoot serum.

### **5.2.3 Stage 1:**

#### **5.2.3.1 Selection of optimum secondary antibody concentrations**

One kangaroo serum sample was randomly selected from the above collection and diluted 1:400 in Tris EDTA buffer with 0.05% Tween 20 (TEN-T, pH 8). One hundred microliters of the diluted serum and 100 µl of TEN-T were added to each well of alternating columns of a Maxisorp flat bottom microtitre plate (Nalge NUNC International, New York). The plate was incubated at 4°C overnight in a humid chamber. The plate was then allowed to equilibrate to room temperature and the serum and buffer were discarded and the plate washed three times with TEN-T. One hundred and fifty microliters of Tris EDTA buffer containing 3 % w/v skim milk powder and 0.05% Tween 20 (TEN-T, pH 8) was added to each well and incubated at 37°C for 60 minutes. The blocking buffer was then discarded and excess buffer removed by gently tapping the plate on absorbent towel. A chequerboard titration of rabbit anti-kangaroo IgG heavy and light chains (Bethyl Laboratories Inc., Montgomery, Texas, USA) and Protein G-HRP were then performed. The plate was incubated at 37°C for 60 minutes in a humid chamber after addition of each antibody and the plate was washed as described previously between incubations. After the final wash 100µl of TMB substrate (Pierce, Thermo Scientific Inc., Rockford, Illinois, USA) was added to each well and incubated at room temperature for 15 minutes and the reaction stopped by adding 100µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plate was read using a BioRad Microplate Reader 6800 (BioRad, Regents Park, New South Wales, Australia) and the final OD of each well determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>. The ratio of the OD values of the wells containing kangaroo serum to the OD values of the wells

without kangaroo serum was calculated. A relatively high ratio was considered to be superior.

#### **5.2.3.2 Evaluating methods of immobilising *Coxiella burnetii* antigen onto the microtitre plate**

Lyophilised phase I and phase II *C. burnetii* antigens (Institut Virion/Serion GmbH, Germany) manufactured for use in the complement fixation test were reconstituted according to the manufacturer's instructions. The 2 antigens were mixed in equal quantities and diluted in carbonate/bicarbonate buffer (pH 9.6) to produce solutions containing doubling dilutions of each antigen. One hundred microliters of each antigen dilution was added to the wells of two columns on two Nunc Maxisorp microtitre plates and the plates incubated in a humid chamber at 4°C overnight. The antigen was discarded from one plate and excess buffer removed by tapping the plate on absorbent towel. The plate was then inverted and allowed to dry at 37°C for 30 minutes. When the first plate was dry the second plate was removed from the humid chamber and the antigen solution discarded and the plate tapped on absorbent towel to remove excess buffer. Each plate was blocked by addition 150 µl of TEN-T containing 3% w/v skim milk powder to each well followed by incubation in a humid chamber for 60 minutes at 37°C. The blocking solution was then discarded and the plates tapped on absorbent towel to remove excess buffer. One hundred microliters of the bovine positive and negative control sera supplied with a CHECKIT Q Fever ELISA diluted 1 in 400 in TEN-T buffer was added to alternating columns and the plates incubated in a humid chamber for 60 minutes at 37°C. Unbound antibodies were removed by washing each plate three times with TEN-T. A titration of Protein G-HRP was then performed. The plate was incubated in a humid chamber for 60 minutes at 37°C after addition of each antibody and the plate was washed as described previously

between incubations. After the final wash 100 µl of TMB substrate was added to each well and incubated for 15 minutes at room temperature and the reaction stopped by adding 100µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plate was read using a BioRad Microplate Reader 6800 and the final OD of each well determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>. For each combination of antibodies the ratio of the OD value for the well containing serum to the OD value of the well with buffer alone was used to compare the two coating methods examined. A relatively high ratio was considered to be superior.

### **5.2.3.3 Confirmation of binding of kangaroo antibodies to *Coxiella burnetii* antigen**

One hundred microliters of the combined phase 1 and phase 2 *C. burnetii* antigen diluted 1 in 100 in carbonate/bicarbonate buffer (pH 9.6) was added to six columns of a Nunc Maxisorp microtitre plate with each column consisting of four wells. In addition, 100 µl of carbonate/bicarbonate buffer containing no antigen was added to one well in each column. The microtitre plate was incubated overnight in a humid chamber at 4°C. The antigen was then discarded and the plates inverted and dried at 37°C for 30 minutes and each well blocked with skim milk powder as described previously. One hundred microliters of 6 different sera diluted 1 in 400 in TEN-T buffer were then added to four-wells in individual columns. The sera were; CHECKiT ruminant positive control; CHECKiT ruminant negative control; and four kangaroo samples (1269, 1270, 1271 and 1420). A solution of rabbit anti-kangaroo IgG heavy and light chains was diluted 1 in 1,000 in TEN-T and 100 µl was added to all wells before incubating in a humid chamber for 60 minutes at 37°C. Washing was performed as described previously and the optimal concentration of protein G-HRP was titrated. The plate was incubated in a humid chamber for 60 minutes at 37°C after addition of each antibody and the



plate was washed as described previously between incubations. After the final wash 100 µl of TMB substrate was added to each well and incubated for 15 minutes at room temperature and the reaction stopped by adding 100µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plate was read using a BioRad Microplate Reader 6800 and the final OD of each well determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>.

#### **5.2.3.4 Reduction of non-specific binding of kangaroo antibodies**

One hundred microliters of the combined phase 1 and phase 2 *C. burnetii* antigen diluted 1 in 100 in carbonate/bicarbonate buffer (pH 9.6) was added to four wells in each of 4 columns of a Nunc Maxisorp. In addition, 100 µl of carbonate/bicarbonate buffer alone was added to one well in each column. The microtitre plate was incubated overnight in a humid chamber at 4°C. The antigen was then discarded and the plates inverted and dried for 30 minutes at 37°C and all wells were blocked with skim milk powder as described previously. For this experiment each serum sample (CHECKiT positive, CHECKiT negative, kangaroo 1274 and kangaroo 1275) was diluted 1 in 400 in each of 1, 2 and 4% w/v skim milk powder in TEN-T and 1, 2 and 4% bovine serum albumin (Sigma, Castle Hill NSW, Australia) in TEN-T or TEN-T alone and allowed to stand at room temperature for 30 minutes. Subsequently, 100 µl of each diluted sample was added to one well of a microtitre plate and incubated in a humid chamber for 60 minutes at 37°C. A solution of rabbit anti-kangaroo IgG heavy and light chains was diluted 1 in 1,000 in TEN-T and 100 µl was added to all wells before incubating in a humid chamber for 60 minutes at 37°C. Washing was performed as described previously following which protein G-HRP concentration was titrated. The plate was incubated in a humid chamber for 60 minutes at 37°C after addition of each antibody and the plate was washed as described previously between

incubations. After the final wash 100µl of TMB substrate was added to each well and incubated for 15 minutes at room temperature and the reaction stopped by adding 100 µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plate was read using a BioRad Microplate Reader 6800 and the final OD of each well determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>.

#### **5.2.3.5 Identification of high-responder kangaroo serum samples and assessing the affect of adding a blocking agent to secondary antibody diluents**

One hundred microliters of the combined phase I and phase II *C. burnetii* antigen diluted 1 in 100 in carbonate/bicarbonate buffer (pH 9.6) was added to all wells of a Nunc Maxisorp microtitre plate. The microtitre plate was incubated overnight in a humid chamber at 4°C. The antigen was then discarded and the plates inverted and dried for 30 minutes at 37°C and all wells were blocked with skim milk powder as described previously. Control sera (ruminant positive and negative controls) and 40 kangaroo test sera (kangaroo samples 1265, 1266, 1268-1285, 1287, 1407-1425) were diluted 1 in 400 in TEN-T plus 4% w/v skim milk powder and mixed on an orbital shaker for 30 minutes at room temperature prior to being added to the previously prepared microtitre plate. One hundred microliters of each control samples was added to four wells on each plate and 100 µl of each test samples was added to duplicate wells. Plates were incubated in a humid chamber for 60 minutes at 37°C. Rabbit anti-kangaroo IgG heavy and light chains antiserum was diluted 1 in 1,000 in TEN-T and 100 µl added to each well and the plate then incubated in a humid chamber for 60 minutes at 37°C. Protein G-HRP was diluted 1 in 8,000 in TEN-T and 100 µl added to each well and the plate incubated in a humid chamber for 60 minutes at 37°C. Washing steps were performed after each incubation as described previously. After the final wash 100 µl of TMB substrate was added to each well and the plate incubated for 15 minutes

at room temperature and the reaction stopped by adding 100 µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plate was read using a BioRad Microplate Reader 6800 and the final OD of each well determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>. Raw data from screening the kangaroo serum samples are presented in Appendix A.

The experiment described above was repeated the following day except 1% w/v skim milk powder was added to the Rabbit anti-kangaroo IgG heavy and light chains antiserum and protein G-HRP diluents immediately prior to use. The OD values from the samples tested without a blocking agent added to the secondary antibodies was compared to the OD values from the test run with a blocking agent added. The difference between the values was expressed as a percentage reduction in absorbance caused by the addition of the skim milk powder. These data are displayed in Appendix A. Visual interpretation of the data from the test results without a blocking agent added to the secondary antibody diluent allowed two populations to be defined based on the samples' relative reactivity. The 'low reacting' sample population had absorbance values less than 1 and the 'high reacting' sample population had absorbance values of 1 or greater. An independent sample t-test was then used to determine if the percentage reduction in absorbance calculated for the 'high reacting' population was significantly different from that calculated for the 'low reacting' population.

#### **5.2.4 Stage 2:**

##### **5.2.4.1 Antigen and antibody titration using high and low kangaroo reactors as controls**

Three kangaroo samples (1278, 1412 and 1417) that were found to have relatively high OD values when tested with the ELISA were pooled and used as a

positive control for this and all subsequent tests. Three kangaroo samples (1274, 1408 and 1418) that were found to have relatively low OD values when tested with the ELISA were pooled and used as a negative control for this and subsequent tests. Raw OD values for these samples are presented in Appendix A. Three separate titrations were performed to determine the optimal concentration of each reagent as follows: antigen was diluted in carbonate/bicarbonate buffer (pH 9.6) and 100 µl added to each well of a Nunc Maxisorp microtitre plate and the plate was incubated overnight at 4°C. The antigen was discarded and excess buffer removed by tapping the plate on absorbent towel. The plate was then inverted and allowed to dry at 37°C for 30 minutes. Each plate was blocked by addition 150 µl of TEN-T containing 3% w/v skim milk powder to each well followed by incubation in a humid chamber for 60 minutes at 37°C. The blocking solution was then discarded and the plates tapped on absorbent towel to remove excess buffer. All incubations with antibodies were performed for 60 minutes at 37°C and were followed by washing as described previously. After the final wash 100µl of TMB substrate was added to each well and incubated for 15 minutes at room temperature and the reaction stopped by adding 100 µl of 1M H<sub>3</sub>PO<sub>4</sub> to each well. The plates were read using a BioRad Microplate Reader 6800 and the final OD of each well was determined by subtracting the OD<sub>570nm</sub> from the OD<sub>450nm</sub>.

Selection of optimum conditions from all titrations was based on the amount of background absorbance observed in negative and blank control wells and the ratio of the OD values of the positive to the OD values of the negative serum with a high ratio was considered to be superior.

#### **5.2.4.2 Titrating control serum against antigen**

In the first experiment the concentration of control serum diluted in TEN-T plus 3% w/v skim milk powder was titrated against the concentration of antigen. For this titration controls containing no serum and no antigen were included and all combinations were tested with rabbit anti-kangaroo heavy and light chain antiserum diluted at 1 in 1000 and 1 in 2000 in TEN-T plus 1% w/v skim milk powder.

#### **5.2.4.3 Titrating rabbit anti-kangaroo antiserum against protein G-HRP**

For this titration the antigen concentration was kept standard at 1 in 50 and serum was diluted 1 in 400 in TEN-T plus 4% w/v skim milk powder. Rabbit anti-kangaroo heavy and light chain antiserum diluted in TEN-T plus 1% w/v skim milk powder was then titrated against protein G-HRP diluted in TEN-T plus 1% w/v skim milk powder.

#### **5.2.4.4 Titrating rabbit anti-kangaroo antiserum against donkey anti-rabbit-HRP**

In the final optimisation experiment antigen concentration was kept standard at 1 in 50 and serum was diluted 1 in 400 in TEN-T plus 4% w/v skim milk powder. Rabbit anti-kangaroo heavy and light chain antiserum in TEN-T plus 1% w/v skim milk powder was then titrated against donkey anti-rabbit-HRP (Bethyl, Montgomery, Texas, USA) diluted in TEN-T plus 1% w/v skim milk powder.

#### **5.2.4.5 Reproducibility of optimised kangaroo Q fever indirect ELISA**

A total of 42 samples of serum that had high OD's (high reactors) and 42 serum samples that had low OD's (low reactors) when tested with the optimised

ELISA were selected and tested once per day for three consecutive days. A one-way ANOVA with Tukey's honestly significant differences test with a 95% confidence limit was performed to determine the statistical significance of any differences in the mean OD values for 'high reacting' and 'low reacting' sets from three consecutive days. The same analysis was also used to test the statistical significance of any differences between the three days when the absorbance results were expressed as a percent of the OD value of the positive control absorbance (PP). All statistical analyses were performed using the SPSS version 15.0 (SPSS Inc., Chicago, USA).

#### **5.2.4.6 Evaluation of an antibody ELISA to detect exposure to *Coxiella burnetii* in serum from western barred bandicoots (*Perameles bougainville*)**

A selection of 36 sera from the western barred bandicoot, (*Perameles bougainville*) bandicoot were tested in exactly the same manner as described for kangaroo serum samples to determine if the immunological test described here could be used for detecting exposure to *C. burnetii* in this marsupial.

#### **5.2.5 Comparison of different cut-off strategies to maximise ELISA performance**

Several strategies were employed to determine the most appropriate method of selecting a cut-off OD that would discriminate between "infected" and "uninfected" kangaroos. A scatter plot was initially constructed to determine if a cut-off OD value could be visually identified. Attempts were made to correlate the data from testing genomic DNA purified from the faeces of each kangaroo with the qPCR (see Chapter 6) and the results from testing the sera from each animal with the ELISA using 5 different cut-off OD values. Spearman's rank correlation was calculated for all cut-offs and qPCR results. The cut-off OD values were

equivalent to; (1) the OD equivalent to 30% of the OD of the positive control serum (30% PP) (OIE, 2000), (2) the OD equivalent to 40% of the OD of the positive control serum (40% PP) (OIE, 2000); (3) the sample OD divided by the mean OD of the negative control samples from the same plate was greater than 1.4 (NEG1.4) and (4) the mean OD of a population of uninfected animals (assumed to be represented by animals sampled from Capel which is discussed in Chapter 6) plus two times the standard deviation of the mean OD (2SD).

#### **5.2.6 Immunological reactivity of kangaroo test sera with phase I or phase II *Coxiella burnetii* antigen individually**

All 343 kangaroo serum samples were tested with an ELISA using each phase of the *C. burnetii* antigen separately diluted 1 in 50 according to the optimised protocol developed here and also described in Chapter 6. The positive and negative kangaroo control sera described previously were also used in this experiment. Each serum sample was diluted 1 in 400 and tested in duplicate and the control samples (positive, negative and no serum) were tested in quadruplicate. The mean OD values of each test sample was normalised by dividing them by the mean absorbance value of the negative controls and then the results were expressed as a ratio of phase I-specific absorbance to phase II-specific absorbance.

A one-way ANOVA was used to determine the statistical significance, at a 95% confidence limit, of any differences between the ratio of OD values using phase I and phase II antigen and four factors; (1) sample collection location (Capel, Manjimup, Badgingarra, Eneabba, Preston Beach and Whiteman Park); animal sex; animal age and the month in which the sample was collected. In addition, samples were classified as positive or negative according to the results

presented in Chapter 6 and the data were analysed to determine differences in relation to the phase I/phase II antigen absorbance ratio. The Poptools Microsoft Excel add-in (Hood 2006) was used to produce summary statistics for the data and to create a frequency histogram.

### **5.3 Results**

#### **5.3.1 Development of an enzyme-linked immunosorbent assay to detect infection with *Coxiella burnetii* in kangaroos: stage 1**

##### **5.3.1.1 Selection of optimum secondary antibody concentrations**

The optimum concentrations of rabbit anti-kangaroo antiserum protein G-HRP and that provided the maximum OD value for the positive control serum and acceptable level of background (OD value of the “no-serum” controls) were 1 in 1,000 and 1 in 8,000 respectively

##### **5.3.1.2 Evaluating methods of immobilising *C. burnetii* antigen onto the microtitre plate**

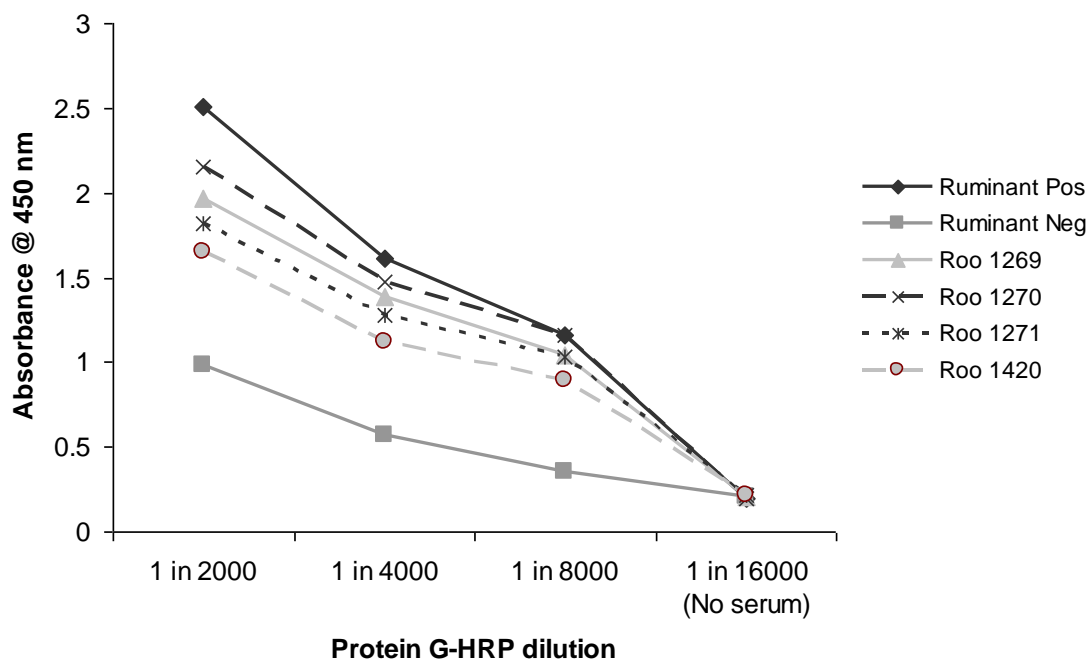
It was determined that drying the plate at 37°C for 30 minutes, after overnight coating at 4°C and then blocking with 3% w/v skim milk powder for 60 minutes at 37°C produced optimum results. These results also indicated that an antigen dilution of 1 in 100 (where each phase of antigen had been diluted 1 in 100) was superior to the other dilutions examined and that a protein G-HRP dilution of 1 in 8,000 was close to optimum.



### 5.3.1.3 Confirmation of binding of kangaroo antibodies to *Coxiella burnetii* antigen

There was a dose-dependent relationship between the OD values for each serum sample and the protein G-HRP dilution. The OD values for all kangaroo samples and the ruminant positive control were greater than the OD value for the ruminant negative control. The relationship between OD values and protein G-HRP dilution for each serum sample is shown in Figure 5.1.

**Figure 5-1. OD values from testing two ruminant control serum samples and four kangaroo serum samples with an ELISA using doubling dilutions of protein G-HRP**



#### **5.3.1.4 Reduction of non-specific binding of kangaroo antibodies**

The addition of blocking agents appeared to have a concentration-dependent effect on the OD values obtained after testing kangaroo serum samples with the ELISA but did not have a similar effect on the ruminant controls. Four percent w/v skim milk powder in TEN-T (pH 8.0) was chosen as the final blocking agent based on the affect that each blocking agent had on the OD values of the positive, negative, kangaroo and blank samples.

#### **5.3.1.5 Identification of high-responder kangaroo serum samples and assessing the affect of adding a blocking agent to secondary antibody diluents**

The raw OD values from testing a panel of kangaroo sera with the ELISA are presented in Appendix A. Adding blocking agent to the diluent for the rabbit anti-kangaroo antiserum and the protein G-HRP produced a marked decrease in the absorbances of all samples. There was a significantly greater change in the OD values caused by the addition of skim milk powder for the 'low reacting' population compared to the 'high reacting' population ( $p < 0.01$ ).

### **5.3.2 Development of an enzyme-linked immunosorbent assay to detect infection with *Coxiella burnetii* in kangaroos: stage 2:**

#### **5.3.2.1 Antigen and antibody titration using high and low kangaroo reactors as controls**

Optimum test performance was achieved when the antigen was diluted 1 in 50 and the serum was diluted 1 in 400. When rabbit anti-kangaroo antiserum was used with protein G-HRP the optimal concentrations of these two antibodies was 1 in 1,000 and 1 in 4,000 respectively. The use of donkey anti-rabbit-HRP in place

of protein G-HRP also reduced background absorbance. A combination of a 1 in 500 dilution of rabbit anti-kangaroo antiserum and a 1 in 4,000 dilution of donkey anti-rabbit-HRP gave the best compromise between low background OD values and high positive control OD values.

#### **5.3.2.2 Reproducibility of optimised indirect ELISA to detect *Coxiella burnetii* in kangaroos**

The mean OD values of 'high reacting' samples tested on day 2 were significantly higher than the absorbance values recorded for the same samples tested on day 1 and day 3 ( $p < 0.01$ ). When the absorbance values for positive samples were converted to a PP it was observed that the values calculated for samples tested on day 3 were significantly higher than the values calculated for samples tested on day 2 ( $p < 0.001$ ). For the 'low reacting' samples it was found that the PP values for samples tested on day 1 were significantly lower than the PP values calculated for samples tested on day 2 ( $p < 0.05$ ).

#### **5.3.2.3 Evaluation of an antibody ELISA to detect exposure to *Coxiella burnetii* in serum from bandicoots**

The results of testing bandicoot sera with the ELISA showed a similar ability to discriminate between high and low responding individuals.

#### **5.3.3 Comparison of different cut-off strategies to maximise ELISA performance**

Significant correlations were found between all ELISA cut-off points applied to the data ( $p < 0.01$ ). However, correlations between qPCR results and the four ELISA cut-offs were not as strong. The JB153-3 qPCR assay was found to

correlate significantly only with the '2SD' cut-off ( $p<0.05$ ) although the IS1111a qPCR correlated weakly with all cut-offs ( $p<0.05$ ) except '30%PP' ( $p>0.1$ ).

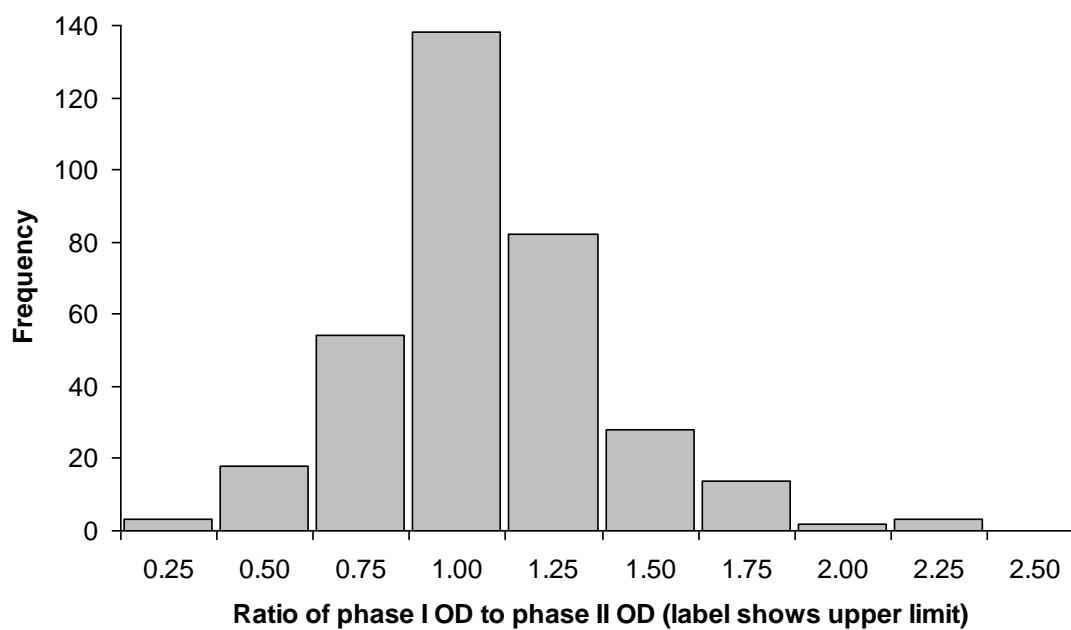
The PP of the samples classified as positive using the '30%PP' cut-off was significantly lower than all other cut-offs employed ( $p<0.05$ ) but no other significant differences were found.

The mean OD value of the kangaroo sera collected in Capel, expressed as a PP, were significantly different from the mean OD values of kangaroo sera from all other collection locations except Badgingarra ( $p<0.01$ ). Of the samples collected from Capel it was found that those collected in July 2007 had significantly higher PP values than those collected in March and May 2007 ( $p<0.05$ ).

#### **5.3.4 Immunological reactivity of kangaroo test sera with phase I or phase II *Coxiella burnetii* antigen individually**

A frequency histogram showing the number of samples displaying phase I/phase II antigen-specific absorbance values is shown in Figure 5.2. One sample (1481) was considered to be an outlier with a ratio of 4.56 and was omitted from further analysis. The mean ratio for samples collected in Capel (1.08) was significantly greater than the mean ratio for samples collected in Manjimup (mean=0.87,  $p<0.01$ ) and the mean ratio of samples collected in Whiteman Park (mean=0.85,  $p<0.05$ ). The ratio for samples collected in March (1.24) was significantly higher compared to the ratio for samples collected in all other months ( $p<0.05$ ).

**Figure 5-2. The ratio of phase I *Coxiella burnetii* antigen OD values to phase II *C. burnetii* antigen OD values for kangaroo sera**



## 5.4 Discussion

### 5.4.1 Assay development

The absence of serum from kangaroos with known *C. burnetii* status complicated the optimisation of the ELISA developed in this study. Initial development with protein G and ruminant control sera enabled a basic assay configuration to be determined that could be applied to kangaroo sera of unknown exposure status. Identification of high and low responder kangaroo sera then allowed the further development and optimisation of the assay.

The assay could be further improved. In particular, attempts could be made to improve the reproducibility of the test, which was relatively poor. It is quite likely that the observed poor reproducibility was caused by using an antigen that was not designed for use in an ELISA. It has been shown that the detection of anti-*C. burnetii* antibodies is greatly affected by the quality of the antigen used (Dane and Beech 1955; Kovacova, Kazar et al. 1998). Unfortunately, no information was available from the manufacturers with regards the method of antigen preparation used. It is reasonable to assume that the antigen is a simple highly purified sample of whole cells and therefore basic mechanical treatment such as sonication (Peter, Dupuis et al. 1987) may expose more antigenic epitopes and thus improve the diagnostic capabilities of this ELISA. However, purification after mechanical treatment may be necessary to avoid masking of specific antigens by cellular debris (Kenny and Dunsmoor 1983). Furthermore, a recent study suggested that antigen made from Nine Mile strain *C. burnetii* is not appropriate for serodiagnosis of coxiellosis in ruminants due to regional differences in strain antigenic reactivity (Rodolakis, Bouzid et al. 2007). Therefore, the Nine Mile strain used in the Virion/Serion antigen may not be representative of the antigenic profile of *C. burnetii* found in Australian marsupials. If a true wildlife-to-domestic animal cycle

exists in Australia then this also has implications in regards to the utility of Nine Mile antigens for surveillance of *C. burnetii* exposure in domestic ruminants in this country. Therefore it is important to isolate and characterise *C. burnetii* from Australian marsupials.

To minimise the affect that the variability of the antigen had on the results, all absorbance values needed to be normalised so that comparisons could be made between samples tested at different times. In most cases this was done by expressing test sample absorbance values as a percentage of the positive control absorbance mean (PP) from the same plate. Initially, statistical analyses were performed on both mean absorbance and PP values but the increased variability seen when the results were expressed in mean absorbance form masked most differences in the comparison.

Further improvement to the ELISA may also be possible by linking a detection enzyme, such as horseradish peroxidase, directly to the rabbit anti-kangaroo IgG heavy and light chain antibodies. This would decrease the duration of the assay by 60 minutes and could lower the amount of background absorbance observed.

It is also encouraging that the indirect ELISA developed as part of this project appears to be compatible with serum from the western barred bandicoot. The relationship between *C. burnetii* and Australian marsupials was established soon after this zoonotic pathogen was first described (Derrick 1939) but only sporadic efforts have been made since to quantify the interaction between them (Dane and Beech 1955; Munday 1972). The availability of an optimised ELISA capable of testing serum from different marsupial species will enable further studies to better characterise the sylvatic and domestic cycles of *C. burnetii* in Australia.

### 5.4.2 Blocking non-specific binding of antibodies

Results from testing the same set of unknown samples with and without the addition of skim milk powder to the secondary antibody diluents showed that the blocking agent had greater effect on the 'low' reactors compared to the 'high' reactors. This is interesting because it indicates that the absorbance produced by high reacting samples is more specific and thus more likely to be indicative of a truly positive test result. Enhancement of signal-to-noise ratio by casein, a component of skim milk powder, is a well established phenomenon utilised in many immunological assays (Kaur, Dikshit et al. 2002; Sentandreu, Aubry et al. 2007). It is in part due to the small size of the casein proteins enabling formation of a tightly packed barrier to subsequently added antibodies (Pratt and Roser). Microtitre plates with high-binding chemical properties, such as the plates used for the assay described here, have been shown to produce high background absorbance in some situations (Rebeski, Winger et al. 1999) and therefore require blocking agents to produce consistent results. However, without verified control sera, preferably from animals infected under controlled conditions, it may be more important to consider the possibility that high absorbance values could in part be due to cross-reactivity with other infectious organisms.

### 5.4.3 Cross-reactivity

Dane and Beech (1955) noted that detection of anti-*C. burnetii* antibodies in kangaroos can be difficult due to the presence of non-specific Wassermann reactivity related to generic antibody-lipopolysaccharide interactions. Cross-reactivity has also been observed between *C. burnetii* and rickettsiae (Field, Mitchell et al. 2000), bartonellaceae (La Scola and Raoult 1996; Numazaki, Ueno et al. 2000) and chlamydiae (Lukacova, Melnicakova et al. 1999). Significantly, in 1999 a new species of *Bartonella* was cultured from the blood of five eastern grey



kangaroos (*Macropus giganteus*) but the pathogenicity and prevalence of this organism are as yet unknown (Fournier, Taylor et al. 2007). Therefore, it would be beneficial to conduct cross-reactivity studies of the kangaroo sera collected as part of this study with antigens from the organisms noted previously.

#### **5.4.4 Selection of a cut-off point for positivity**

The fully optimised assay described here was able to provide distinction between those samples that produced strong reactivity to the antigen and those with a weak association with the antigen. However, selecting a cut-off value that best separates these two populations and therefore provides optimum sensitivity and specificity is difficult. If a bank of known-negative samples is available then a cut-off can be calculated using the mean negative sample absorbance plus two or three standard deviations of the mean, depending on the sensitivity and specificity desired (Greiner, Kumar et al. 1997). While the true infection status of animals in this study was unknown, the animals sampled in Capel exhibited significantly lower reactivity for the antigen than the other sampling locations. However, of the samples taken from Capel, variation in regards to the month in which the samples were collected was observed. It is reasonable to define the samples collected in March and May as a negative population of animals based on the more extensive analysis of the factors that are related to the reactivity of the kangaroo serum samples (Chapter 6).

Using this putatively negative population to determine a cut-off value may have been confounded by the poor reproducibility of the test. If a set OD value is used to determine the exposure status of an individual sample, the variability caused by the antigen may have a significant effect on the results of the test. In the absence of a known negative population the positive controls used in the test

can serve as a benchmark from which positivity can be inferred for unknown samples. Normalising test serum absorbance results against positive control absorbance and then implementing a cut-off at a certain percentage of the positive control mean has been used in research and commercially (OIE 2000) and this approach appeared to separate the sample population absorbance values quite well. However, the positive controls used for all testing consisted of a pooled sample of previously identified high reacting sera that were not confirmed as positive by any other means due to a lack of appropriate tests. Similarly, using the pooled negative control absorbance mean as a standardising point for test sample results may not be applicable to the wider population of kangaroos in Australia.

When the various cut-offs were compared with an analysis of variance it was found that they were not significantly different, with the exception of the test in which the cut-off value was 30% of the positive control absorbance. This cut-off was shown to give more 'positive' results than the other cut-off values tested. The '40%PP' and 'NEG1.4' cut-offs correlated well with each other and were found to divide the samples into 'positive' and 'negative' populations that had mean PP values that were not different from each other. In most respects these two cut-offs can be considered to be equivalent and either would be appropriate for classification of exposure status for kangaroo serum samples until confirmed control sera are available. However, given that the use of a '40%PP' cut-off is supported in the literature (OIE 2000; Rebeski, Winger et al. 2001; Giammanco, Chiarini et al. 2003; Hoet, Chang et al. 2003; Nielsen, Toft et al. 2004) and in commercial kits such as the CHECKiT Q fever ELISA as being a valid method for discriminating positive and negative samples, it was chosen for application to the kangaroo test samples. It may also be beneficial to define an equivocal range within which tested samples have absorbance values close to, but not exceeding, the cut-off for positivity (Giammanco, Chiarini et al. 2003). Such samples are

considered to be 'suspect' and are subsequently re-tested. A second 'suspect' result leads to the sample being classified as 'negative' and 'negative' or 'positive' results are considered to be definitive. This approach may be especially useful for the test described here until further development of the antigen can be undertaken to improve reproducibility.

#### **5.4.5 Specific reactivity of sera to phase I or II antigen**

Significant effort has been made to identify factors associated with *C. burnetii* that are predictive of either chronic or acute Q fever in humans. Molecular methods have met with debatable success (Yu and Raoult 1994; Toman, Hussein et al. 2003) but the correlation between immunological factors and disease syndrome is much more widely accepted. In humans it has been demonstrated that antibodies to phase II antigen predominate in the early stages of infection, whereas anti-phase I antibodies appear somewhat later (Hunt, Field et al. 1983; Peacock, Philip et al. 1983; Peter, Dupuis et al. 1985) and a high proportion of anti-phase I antibodies has been shown to indicate a chronic infection (Wilson, Neilson et al. 1976; Hunt, Field et al. 1983; Peacock, Philip et al. 1983; Tissot-Dupont, Thirion et al. 1994). This pattern has also been demonstrated in guinea pigs (Moos and Hackstadt 1987) and it seems possible that the same temporal relationship between infection stage and phase-specific antibodies exists for other host species.

While significant differences were observed when comparing the different collection locations it was not possible to draw any strong conclusions because of a lack of data for all locations over all months. Perhaps the most significant trend revealed is that, of all samples, those collected in March had a significantly higher phase I to phase II ratio than any other month. If the immune response

progression in kangaroos is similar to that observed for humans and guinea pigs these results could indicate that chronic infection predominated in March and earlier while there was a high rate of new or acute infections in months after March. The implications of these observations are presented in the discussion in Chapter 6.

#### **5.4.6 Conclusions**

In summary, the ELISA discussed in this chapter is the first optimised species-specific immunological assay described for the detection of *C. burnetii* exposure in kangaroos. Furthermore, the test appears to be compatible with other marsupial species and therefore could be an important epidemiological tool for surveillance of *C. burnetii* in these native Australian species. Development of the assay using a generic-specificity secondary antibody allows scope for adaptation of the test to other species which could be important for the dissemination of *C. burnetii* in Australia. Of particular interest is the feral pig population which often occupies locations close to water catchments for human consumption (DAFWA 2008). The ELISA is also capable of providing important information regarding the specificity of the hosts' antibody response to phase I or phase II antigen and thus, by inference, whether the animal tested has acute or chronic infection. Applying this approach to the kangaroo samples collected for this project seemed to reveal that the faecal-oral route may be important in transmission of the pathogen.

## **6. A survey of Western Australian sheep, cattle and kangaroos to determine the prevalence of *Coxiella burnetii***

### **6.1 Introduction**

The agent responsible for Q fever, *Coxiella burnetii*, has a strong association with domestic ruminants, including sheep, goats and cattle, (Raoult and Marrie 1995) as well as native Australian marsupials (Derrick 1939; Pope, Scott et al. 1960). Interaction with domestic livestock has been implicated as a significant factor contributing to *C. burnetii* seroconversion in wildlife species (Enright, Franti et al. 1971) and this domestic animal to wildlife transfer may represent a permanent cycle for maintenance of *C. burnetii* in the environment (Dane and Beech 1955). There have been no published surveys on the prevalence of Q fever in domestic or native animals in Western Australia.

Serological detection of exposure to *C. burnetii* in ruminants is well established using complement fixation (Field, Mitchell et al. 2000; Rousset, Durand et al. 2007), immunofluorescence (Setiyono, Ogawa et al. 2005; Rousset, Durand et al. 2007) and enzyme linked immunosorbent assays (Masala, Porcu et al. 2004; Berri, Crochet et al. 2005). Likewise, polymerase chain reaction-based assays have been used for detecting *C. burnetii* DNA in faeces and other bodily excreta from livestock (Berri, Laroucau et al. 2000; Arricau Bouvery, Souriau et al. 2003; Guatteo, Beaudeau et al. 2006).

There are four main groups of kangaroos in Australia; the eastern and western grey kangaroo group, the red kangaroo, the antelope kangaroo and the wallaroo-euro group (Dawson 2002). In Western Australia the western grey (*Macropus fuliginosus*) and the red kangaroos (*M. rufus*) are most common and are harvested

commercially. The western grey kangaroo has flourished with the introduction of European farming methods and often grazes the same pastures as domestic livestock while the Red kangaroo prefers more arid conditions (Management 2002).

This study was designed to apply immunological and molecular methods to determine the prevalence of *C. burnetii* in cattle, sheep and kangaroos in Western Australia.

## **6.2 Materials and methods**

### **6.2.1 Collection of serum and faeces or urine from sheep, cattle and kangaroos in Western Australia**

A map showing the approximate locations of sampling sites is presented in Figure 6.1. The map was generated using the Magellan Discover AUS Streets and Tracks software (Magellan, San Dimas, California, USA).

**Figure 6-1. Map of the approximate locations of sampling sites for collection of serum and faeces from cattle, sheep and kangaroos in Western Australia (adapted from a map generated in the Magellan Discover AUS software)**



### 6.2.1.1 Bovine samples

Paired samples of blood and faeces were collected from 124 cattle held at a feedlot in Vasse, Western Australia (WA) in 2007. The herd comprised approximately 80% *Bos indicus* steers (except for one heifer) from three properties in the North West of WA and 20% Angus steers (*B. taurus*) from the South West of WA. All animals were between 18 and 24 months of age.

Paired blood and urine samples were also collected from 157 *B. taurus* cattle from a farm in Pinjarra, WA that had experienced an outbreak of leptospiral abortions in 2005 (Wai'in 2007). A further 48 paired faecal and blood samples were collected from mixed age *B. taurus* cattle housed on the Murdoch University farm in February 2007.

### 6.2.1.2 Ovine samples

Paired faecal and blood samples were collected in February 2007 from 50 merino ewes (*Ovis ovis*) approximately 5 years of age. These animals were used for breeding purposes on the Murdoch University farm. Samples were collected when the animals were not pregnant and had not lambed recently.

### 6.2.1.3 Kangaroo samples

Paired blood and faecal samples were taken from western grey kangaroos from various locations in the South West Western Australia including: Preston Beach, Whiteman Park, Manjimup, Capel, Badgingarra and Eneabba (see Fig. 6.1). The approximate age of each animal sampled was recorded as either '1' (pouch young), '2' (juvenile), '3' (sub-adult) or, '4' (adult). Pouch young represented those joeys too young to leave the pouch, whilst juveniles represented those young at foot who could return to the pouch at will. Sub-adults were



considered to be those kangaroos who had not yet reached mature body weight, compared to adults, who were considered fully grown.

### **6.2.2 Detection of antibodies to *Coxiella burnetii* using the complement fixation test**

Serum from all ruminants and a preliminary selection of kangaroos was tested using the CFT by the Department of Agriculture and Food Western Australia (DAFWA) according to their in-house procedures. All samples that reacted strongly at a 1 in 8 dilution were heat-inactivated at 58°C for 30 minutes and subsequently re-tested.

### **6.2.3 Detection of antibodies to *Coxiella burnetii* in serum from ruminants from Western Australia using an ELISA**

Sera were tested using the CHEKiT Q Fever ELISA kit (IDEXX Laboratories Inc., Switzerland) according to the manufacturers' instructions. A sample was considered positive if the optical density (OD) was  $\geq 40\%$  of the mean OD value of the positive control wells for that plate. Samples with OD values less than 30% of the positive control values were considered negative and samples with OD values between 30 and 40% of the positive controls were considered suspect and were re-tested. Samples that were re-tested were evaluated in the same way except that if the OD value of a sample was in the "re-test" range the sample was classified as negative.

#### **6.2.4 Detection of antibodies to *Coxiella burnetii* in serum from kangaroos from Western Australia using an ELISA**

The antibody-ELISA described in Chapter 5 was used to detect antibodies to *C. burnetii* in kangaroo serum. All samples were tested in duplicate and the OD values of the samples were normalised by dividing them by the mean OD value of the positive control from the same microtitre plate and converted to a percentage (PP). All samples with PP values  $\geq 40\%$  were classified as positive.

#### **6.2.5 Purification of *Coxiella burnetii* whole genomic DNA from faeces and urine**

Whole genomic DNA was extracted from all marsupial faecal samples and most ruminant samples using the optimised procedure described in Chapter 2. The DNA purified from bovine urine were kindly provided by Dr. Peter Wai'in. The method used was as follows. Five millilitres of each sample was centrifuged for 30 minutes at  $3,000 \times g$  and all but 0.5 ml of the supernatant was discarded. The pellet was resuspended in the retained supernatant and centrifuged for 10 minutes at  $7,500 \times g$  and the whole supernatant was carefully removed. Whole genomic DNA was then extracted according to one of two methods. For clear urine samples the pellet was resuspended in 50  $\mu$ l of sterile water by vigorous pipetting, incubated at room temperature for two minutes then incubated at 95°C for 10 minutes. Tubes were centrifuged briefly to condense droplets in the cap prior to storage at -20°C. This method was obtained from the Animal Research Institute, Queensland Department of Primary Industry and Fisheries, Moorooka, Queensland. For urine samples that were contaminated by faecal matter a Qiagen Tissue Minikit (Qiagen, Hilden, Germany) was used to extract and purify whole

genomic DNA according to the manufacturers instructions with the final elution made in 50 µl of buffer AE.

#### **6.2.6 Quantitative PCR detection of *Coxiella burnetii* DNA isolated from faeces and urine**

*Coxiella burnetii* DNA was amplified using two qPCR assays as described in Chapter 2 with 5 µl of DNA template per reaction. All samples were tested in duplicate and two 'no template' controls (NTC) were included with every run. Four *C. burnetii* DNA standards made from Q Vax™ vaccine (CSL, Parkville, Australia) as described previously were also included in each run to enable quantification of target DNA in unknown samples.

#### **6.2.7 Conventional PCR and sequencing**

*Coxiella burnetii* DNA was also amplified using a conventional PCR with the OMP1/OMP2 primer set performed as described previously (Zhang, Nguyen et al. 1998). Briefly, reaction mixes contained 200 mM dNTP's, 1.5 mM magnesium chloride, 2.5 µl of 10x reaction buffer, 5 Units of TAQ-1 DNA polymerase (Fisher Biotech, Perth, Western Australia), 20 pmoles of each primer and PCR-grade water to a total volume of 23 µl. Two microliters of purified DNA template was added to each reaction mix. Cycling conditions consisted of an initial denaturation step at 94°C for 3 minutes followed by 40 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute. Cycling was performed on an Applied Biosystems GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, California, USA).

The purified PCR products were sequenced using the Big Dye version 3.1 terminator kit (Applied Biosystems) using the dideoxynucleotide chain termination method (Sanger, Nicklen et al. 1977). The sequence was determined using an

ABI Prism Applied Biosystems 377 automatic DNA sequencer (Applied Biosystems) at the State Agriculture and Biotechnology Centre (Perth, Western Australia). Chromatogram sequencing files were edited using Chromas Lite version 2.0 (Technelysium P/L, Helensvale, Queensland, Australia). Sequence information obtained was compared to sequence information previously submitted to GenBank using BLAST software available from <http://www.ncbi.nlm.nih.gov>.

Sequencing was performed on all samples that were positive when tested with the JB153-3 qPCR and a random selection of samples that were positive when tested with the *IS1111a* qPCR. Sequencing was attempted on amplicons from 13 ruminant samples and 16 kangaroo samples.

#### **6.2.8 Assessment of the effect of PCR-inhibitors in faecal DNA extractions**

Samples of DNA from faeces that produced no detectable amplification were used to assess the amount of PCR inhibition caused by factors that were co-purified during the DNA extraction process. Forty two bovine samples, 20 ovine samples and 38 marsupial samples were selected for this experiment. Eighteen micro litre aliquots were taken from each sample and 2 µl of DNA extracted from Q-Vax vaccine using the Qiagen Tissue Minikit was added. Two microliters of DNA extracted from Q-Vax vaccine was also added to 18 µl of high-pure water to serve as an uninhibited control. Amplification of 5 µl of template per reaction, in duplicate, was conducted and data analysed as described previously.

The statistical significance of any differences in the estimate concentration of genome equivalents present in control and test samples was determined using a one-way ANOVA and post-hoc (LSD) analysis at a 95% confidence limit.

### 6.2.9 Isolation of *Coxiella burnetii* from bovine and kangaroo faeces

Isolation of viable *C. burnetii* cells was attempted with four ruminant and six kangaroo samples. All in vitro culture work was conducted by Michelle Lockhart in the PC-3 biocontainment laboratory [AQIS approved] of the Australian Rickettsial Reference Laboratory (ARRL), in the Hunter Area Pathology Service, John Hunter Hospital, Newcastle, NSW. In the procedure developed by the ARRL faecal samples were resuspended in 10 ml of PBS and mixed until the suspension was homogenous. Solid matter was removed by centrifugation at  $100 \times g$  for five minutes and the resulting supernatant was centrifuged at  $5,000 \times g$  for 15 minutes to pellet bacterial cells which were subsequently resuspended in 5 ml of PBS. The suspensions were subjected to two further slow speed/high speed centrifugation steps ( $100 \times g/5,000 \times g$ ) before passing the resuspended pellet through a  $0.45 \mu\text{m}$  filter. Half of the filtrate was divided into two confluent cultures of vero cells and two microfuge tubes for DNA extraction according to the procedure described by Klee and company (2006). The remaining filtrate was passed through a  $0.22 \mu\text{m}$  filter and the resulting liquid was again divided equally between two flasks of vero cells and two microfuge tubes for DNA extraction according to the procedure of Klee and company (2006). Faecal samples had been frozen after collection and subjected to at least three freeze-thaw cycles prior to attempts at isolation.

### 6.2.10 Data analysis

All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS version 15.0, SPSS Inc., Chicago, USA) unless indicated otherwise.

Analysis of raw qPCR data was performed using the Rotorgene<sup>®</sup> 3000 software to automatically select optimal cycle threshold cut-offs based on the slope

of the standard curve and the  $R^2$  value. The user-defined DNA concentration of the standards was then used by the software to provide estimates of the DNA concentration of the unknown samples.

The level of agreement between the immunological tests and qPCR was determined by calculation of the kappa statistic (Petrie 1999). A two-tailed dependent sample Student's T-test was also used to determine the statistical significance of any difference in genome equivalents estimated using the IS1111a qPCR and JB153-3 qPCR with samples classified as either positive or negative using data from the ELISA results.

Due to the low number of immunologically-positive ruminant samples no statistical analyses were performed using these data. The apparent prevalence and 95% binomial confidence intervals (Petrie and Watson, 1999) were calculated using results from testing bovine and ovine serum and faeces with ELISA and qPCR respectively.

A one-way ANOVA with post-hoc analysis (Tukey's HSD) and two-tailed independent sample Student's T-test with 95% confidence limits were used where appropriate to determine the statistical significance of observed differences in the mean genome equivalent copy number estimated using results from the qPCR and ELISA for ruminant and kangaroo samples and other factors including: collection location and month, age and sex.

## **6.3 Results**

### **6.3.1 Results from testing ruminant and kangaroo serum for the presence of anti-*Coxiella burnetii* antibodies**

#### **6.3.1.1 Results of testing sera for the presence of anti-*Coxiella burnetii* antibodies using the complement fixation test**

All bovine and ovine serum samples were negative when tested with the CFT. None of the sera reacted strongly at a dilution greater than 1 in 8. No strong reactions were seen in any of the samples that were heat inactivated.

A random selection of 20 serum samples from kangaroos was tested using the CFT to determine if the test was applicable to this species. Strong non-specific reactions that could not be reduced through heat inactivation were observed in all samples and this made interpretation of results impossible.

#### **6.3.1.2 Detection of antibodies to *Coxiella burnetii* in serum from using an ELISA**

Two of the 329 bovine sera (0.6%; 0-1.4% CI's) and no ovine sera were positive using the CHEKiT Q fever ELISA based on the cut-off specifications recommended by the manufacturer. Four bovine sera were classified in the 'suspect' range (between 30% and 39% of the absorbance value for the positive control wells) when initially tested. All of these samples were re-tested and none met the criteria for positivity and thus were classified as negative. One of the two positive samples was collected from an animal in Pinjarra while the other was taken from an animal at the Vasse experimental feedlot, which had originally been purchased from a farm in the Gascoigne region of WA.

### **6.3.1.3 Detection of antibodies to *Coxiella burnetii* in serum from kangaroos using an ELISA**

A total of 115 of 343 (33.5%; 28.5-38.5% CI's) sera from kangaroos were positive when tested using the modified ELISA. The number of ELISA-positive samples from animals from each location, the number of ELISA-positive samples for each sex and age group of kangaroo and the number of ELISA-positive samples for each collection month are shown in Tables 6.1-6.3 respectively.



**Table 6-1. The number of ELISA-positive serum samples from kangaroos from different locations in Western Australia (95% confidence intervals in parentheses)**

<b>Location</b>	<b>Number positive</b>	<b>Number sampled</b>	<b>Percent positive (95% CI's)</b>
<b>Capel</b>	10	123	8.13 (3.3, 13.0)
<b>Manjimup</b>	43	113	38.05 (29.10, 47.01)
<b>Bagingarra</b>	12	30	40.00 (22.47, 57.53)
<b>Preston Beach</b>	23	28	82.14 (67.96, 96.33)
<b>Eneabba</b>	10	17	58.82 (35.43, 82.22)
<b>Whiteman Pk</b>	17	32	53.13 (35.83, 70.42)
<b>TOTAL</b>	115	343	33.53 (28.53, 38.52)

**Table 6-2. The number and proportion of ELISA-positive kangaroo serum samples for each sex and age group (95% CI's in parentheses)**

		Number positive	Number sampled	Percent positive (95% CI's)
<b>Sex</b>	Male	64	177	36.2 (29.1, 43.2)
	Female	51	166	30.7 (23.7, 37.7)
<b>Age</b>	1	0	3	0.0
	2	2	4	50.0 (1.0, 99.0)
	3	12	40	30.0 (15.8, 44.2)
	4	101	296	34.1 (28.7, 39.5)

**Table 6-3. The number and proportion of ELISA-positive kangaroo serum samples for each collection month (95% CI's in parentheses)**

<b>Month</b>	<b>Number positive</b>	<b>Number sampled</b>	<b>Percent positive (95% CI's)</b>
<b>March</b>	0	36	0.0
<b>April</b>	0	20	0.0
<b>May</b>	22	65	33.9 (22.3, 45.4)
<b>June</b>	61	138	44.2 (35.9, 52.5)
<b>July</b>	32	84	38.1 (27.7, 48.5)

Serum from three pairs of females and their pouch young (young number 1433 with mother 1434, young 1594 with mother 1604 and young 1595 with mother 1600) were tested with the modified ELISA (using combined phase I and phase II antigen) and with phase I and phase II antigen individually as described in Chapter 5. For the first pair no antibodies were detected in either mother or young using the combined *C. burnetii* antigens and the ratios of pl/plI for mother and young were 1.18 and 0.66 respectively. For the second and third mother/young pairs the mothers were ELISA-positive but the pouch young were negative. All serum samples from pouch young had OD values below the negative control values when tested with the ELISA.

The mean PP values of samples collected in June were significantly higher than the PP means of samples collected in March, April and May ( $p < 0.05$ ). The mean PP values of samples collected in July were significantly higher than the PP values of samples collected in March and April ( $p < 0.005$ ).

There was no significant difference in the mean PP values of male and female kangaroos and there was no significant association between the calculated PP values and the age group of the kangaroos.

The mean PP for samples collected in Capel was significantly lower than the PP for samples collected in all other locations with the exception of Badgingarra ( $p < 0.001$ ). The mean PP values of samples collected from Manjimup and Badgingarra were significantly lower than the PP values of samples collected at Preston Beach ( $p < 0.05$ ).

The mean PP values for samples collected from Capel in March and May were found to be significantly lower than the mean PP values for samples collected in

July ( $p < 0.05$ ). No statistically significant association was found between the mean PP values and the sex of the animals sampled in Capel.

The mean PP values of samples collected from Manjimup in April were significantly lower than the mean PP of samples collected in other months ( $p < 0.001$ ). No statistically significant association was found between the mean PP values and the sex of the animals sampled in Manjimup.

### **6.3.2 Results from testing ruminant faecal and urine samples for the presence of *Coxiella burnetii* DNA using a quantitative polymerase chain reaction**

The number of positive bovine and ovine samples in each collection location according to the IS1111a qPCR tests are shown in Table 6.5. As there were only three ruminant samples that were positive when tested with the JB153-3 qPCR these results have been excluded from the table. Table 6.6 shows the number of IS1111a qPCR-positive samples that were detected in each collection location, table 6.7 shows the number of IS1111a qPCR-positive samples that were detected for each sex and age group of kangaroo and table 6.4 shows the number of IS1111a qPCR-positive samples that were detected for each collection month. As there were only six kangaroo samples that were positive when tested with the JB153-3 qPCR these results have been excluded from the following tables.

**Table 6-4. Results from testing faeces and urine collected from cattle and sheep from different collection locations with the IS1111a qPCR**

		<b>Species</b>	
		<i><b>Bovine</b></i>	<i><b>Ovine</b></i>
<b>Pinjarra</b>	No. Tested	113	NA
	No. Pos	14	NA
	%	12.4	NA
<b>Northern</b>	No. Tested	124	NA
	No. Pos	11	NA
	%	8.9	NA
<b>Murdoch</b>	No. Tested	48	50
	No. Pos	1	6
	%	2.1	12

**Table 6-5. Results from testing faeces from kangaroos from different locations with the IS1111a qPCR (95% CI's in parentheses)**

	Number positive	Number sampled	Percent positive (95% CI's)
<b>Capel</b>	10	123	8.1 (3.3, 13.0)
<b>Manjimup</b>	17	113	15.0 (8.5, 21.6)
<b>Bagingarra</b>	3	30	10.0 (-0.7, 20.7)
<b>Preston Bch</b>	1	28	3.6 (-3.3, 10.5)
<b>Eneabba</b>	2	17	11.8 (-3.6, 27.1)
<b>Whiteman Pk</b>	8	32	25.0 (10.0, 40.0)
<b>TOTAL</b>	41	343	123.0 (8.5, 15.4)

**Table 6-6. Results from testing faeces from kangaroos for each sex and age group with the IS1111a qPCR (95% CI's in parentheses)**

		Number positive	Number sampled	Percent positive (95% CI's)
	Male	26	177	14.7 (9.5, 19.9)
<b>Sex</b>	Female	15	166	9.0 (4.7, 13.4)
	1	0	3	0.0
	2	0	4	0.0
	3	6	40	15.0 (3.9, 26.1)
<b>Age</b>	4	35	296	11.8 (8.2, 15.5)



**Table 6-7. Results from testing faeces from kangaroos for each collection month with the IS1111a qPCR (95% CI's in parentheses)**

	Number positive	Number sampled	Percent positive (95% CI's)
<b>March</b>	8	36	22.2 (8.6, 35.8)
<b>April</b>	5	20	25.0 (6.0, 44.0)
<b>May</b>	8	65	12.3 (4.3, 20.3)
<b>June</b>	11	138	8.0 (3.5, 12.5)
<b>July</b>	9	84	10.7 (4.1, 17.3)

#### **6.3.2.1 Inhibition of PCR by factors co-purified during the DNA isolation procedure**

Five of the 42 bovine samples of faeces tested contained factors that significantly reduced the estimated DNA concentration ( $p < 0.05$ ) in a qPCR system. None of the 20 ovine extracts tested displayed inhibition of amplification that was significant at the 95% confidence level, while five of the 32 kangaroo faecal extracts had significantly lower estimates of *C. burnetii* DNA concentration ( $p < 0.05$ ) than the mean DNA concentration of all samples.

#### **6.3.2.2 Results from testing ruminant faecal and urine samples for the presence of *Coxiella burnetii* DNA using a quantitative polymerase chain reaction**

All no template controls were negative in all runs of the PCR. No significant differences were found when comparing sample result classification and collection location or ruminant species.

#### **6.3.2.3 Results from testing kangaroo faecal samples for the presence of *Coxiella burnetii* DNA using a quantitative polymerase chain reaction**

No template controls were negative in all runs of the PCR. There were no significant associations observed in the proportion of all samples positive using the JB153-3 and IS1111a qPCR tests and the origin, age and sex of the kangaroos and month of sample collection. There were significantly more qPCR-positive samples detected from animals sampled in March in Capel compared to May and July ( $p < 0.05$ ).

#### 6.3.2.4 Sequencing results

Sequencing of the *com1* amplicon was successful in all but one instance with all matches showing greater than 99% identity with *C. burnetii* Dugway strain. A summary of the sequence results is shown in Appendix B.

#### 6.3.3 Isolation of *Coxiella burnetii* from faeces

Attempts to isolate *C. burnetii* from four ruminant and six kangaroo faecal samples were unsuccessful. Samples were re-tested with the JB153-3 qPCR which confirmed the presence of *C. burnetii* DNA.

### 6.4 Discussion

#### 6.4.1 Testing bovine and ovine serum samples for the presence of anti-*Coxiella burnetii* antibodies using the complement fixation test and ELISA

There are many published reports that confirm that *C. burnetii* is endemic in the world-wide ruminant population with greater than 19% of animals showing an immunological response indicative of exposure (Arricau-Bouvery and Rodolakis 2005). There is also evidence suggesting that prevalence of exposure to *C. burnetii* is increasing in some countries (Hellenbrand, Breuer et al. 2001; Hatchette, Campbell et al. 2002). Despite this there is little data on the prevalence of *C. burnetii* infection in domestic livestock in Australia. Indeed, one of the few studies in Australia suggested that ruminant livestock in South Australia were not a significant reservoir of *C. burnetii* infection (Dane and Beech 1955). This finding was supported by a serological survey of abattoir workers in Queensland that found that only 1% of individuals had detectable antibodies against *C. burnetii* (McKelvie 1980). These studies were conducted over 28 years ago and so the data provided and the methods used are not current. However, the results of this

study indicate that the conclusions of Dane and Beech (1955) and McKelvie (1980) may still be relevant.

Results from testing 379 ruminant samples with the CFT and CHEKIT Q fever ELISA showed low to negligible sero-prevalence. It is possible that the low apparent prevalence of *C. burnetii* infection may be due to the poor sensitivity of the tests used. It has been found that the sensitivity of CFT (73%) is inferior to that obtained in an ELISA (99%) but the CFT has superior specificity (90%) in comparison to ELISA (88%) (Field, Mitchell et al. 2000). However, it is unlikely that the population of ruminants sampled had a true seroprevalence any higher than 2%. It is also possible that using antigen made with the Nine Mile strain of *C. burnetii* may not be appropriate for use in Australian studies and may lead to an underestimation of the serological prevalence of this pathogen (Rodolakis, Bouzid et al. 2007). However, the animals in this study had diverse points of origin prior to being sampled and thus the results presented here may represent a more widespread trend in the state of Western Australia.

#### **6.4.2 Testing ruminant faecal and urine samples for the presence of *Coxiella burnetii* DNA and the agreement of these results with immunological findings**

Sequencing of the OMP1/OMP2 PCR products gave results that were most similar to the Dugway *C. burnetii* strain. Previous studies have placed an Australian human isolate of *C. burnetii* in genetic Group II while the Dugway strain has been placed in Group VI according to RFLP analysis (Hendrix, Samuel et al. 1991). However, a more recently published study found that, on the basis of 16S rRNA and *IS1111a* DNA sequence comparisons, a series of wombat isolates showed greatest similarity to the Dugway strain (Vilcins, Old et al. 2009). Further genetic characterisation of *C. burnetii* DNA in positive kangaroo faecal samples

may provide valuable insights into the relatedness of Australian isolates of *C. burnetii*.

There was poor agreement between the ELISA results and the qPCR targeting the *IS1111a* repetitive element, which detected a significantly higher proportion of test- positive samples. This may be because in the early stages of *C. burnetii* infection the host may not have generated an immune response to *Coxiella* but may still be shedding organisms in bodily secretions and faeces (Berri, Souriau et al. 2002). The sensitivity of the Idexx CHEKIT ELISA has also been questioned (Rodolakis, Berri et al. 2007) and this must be taken into account when interpreting these data. However, the issue of contamination of the qPCR samples should also be considered. Contamination could have occurred before or after DNA extraction with either PCR product or with *C. burnetii* cells themselves (Kwok and Higuchi 1989). The likelihood of contamination of new reaction mixtures with PCR product was reduced through the use of Invitrogen Platinum qPCR master mix (refer to Chapter 2 for details) and confirmatory testing with qPCR assays targeting other *C. burnetii* genes. Results that show a positive classification of samples in only one assay supports the theory that contamination was not caused by carryover of PCR amplicon. Therefore, if contamination was causing false positive PCR and sequencing results it was most likely due to whole phase II *C. burnetii* cells (only the phase II form was cultured at Murdoch University) and the distinction between a true and false result would not be possible with the gene targets used to detect the organism. Thus, the quantitative PCR that was developed to amplify phase I *C. burnetii* only, JB153-3, may provide a more accurate representation of the samples that were truly positive. However, the *JB153-3* gene lies in a redundant genomic region that is not under the same selection pressure as genes which are transcriptionally active. It is possible that wild-type strains may not have retained this genetic element although the *JB153-3*

element was present in the genomes of all four phase I strains tested and absent from the four phase II strains of *C. burnetii* that have been analysed (Hoover, Culp et al. 2002). If the JB153-3 qPCR results are used to identify the positives then the confidence in a positive result increases and the confidence in a negative result decreases. These results also highlight the extreme contamination risk that *C. burnetii* poses in laboratories where *in vitro* culture and ultra-sensitive diagnostic qPCR are performed side-by-side.

### 6.4.3 Ruminants and Q fever in Australia

The finding that the prevalence of antibodies to, and shedding of, *C. burnetii* by the ruminants sampled is at odds with epidemiological studies from other countries. However, it is almost certain that cattle, sheep, and to a lesser extent, goats are involved in transmission of *C. burnetii* to humans in Australia. Many human cases are reported every year in red meat industry workers and it is a recognized occupational hazard in this group (Garner, Longbottom et al. 1997; Worksafe 2001; Chong, La Brooy et al. 2003). A retrospective cohort study in Australia showed that of 2555 abattoir workers who were vaccinated with Q-Vax, two cases of Q fever were reported and both of these were within a few days of vaccination (Ackland, Worswick et al. 1994). Conversely, Ackland et al. (1994) noted that there were 55 cases among the 1365 unvaccinated employees. The results of the study by Ackland et al. (1994) indicated that vaccination appeared to be protective for at least five years (the period of the study) although repeated natural exposure may have contributed to protection. Therefore, the mandatory vaccination program in the red meat and kangaroo abattoirs in some Australian States appears to be justified, but the interaction between domestic and wildlife cycles of *C. burnetii* in Australia remains unknown.

#### **6.4.4 Detection of antibodies to *Coxiella burnetii* in serum from kangaroos from Western Australia using an ELISA**

Results from testing serum from kangaroos from WA suggest that Australian marsupials may play a more significant role in the maintenance of *C. burnetii* in the environment than was previously thought. The ELISA results suggest an exposure rate of nearly 34% in all collection locations spanning approximately 500 kilometres from the most northerly location to the location furthest south. In some areas the prevalence of anti-*C. burnetii* antibodies was as high as 92.8%, although there was a strong relationship between sero-positivity and the month that the samples were collected which probably skewed the results for locations that were only sampled once or twice. Samples collected in Capel did not yield test results that were significantly influenced by month whereas those collected in Manjimup did, indicating that seropositivity of kangaroos may also be linked to the home range of a particular population. The results support earlier work in Australia (Pope, Scott et al. 1960) that found that 68 of 296 kangaroos sampled were antibody positive for *C. burnetii* according to the CFT and 44 of 296 were agglutinin positive. Pope et al. (1960) observed that red kangaroos had a higher incidence of complement fixing antibodies than did grey kangaroos and in some locations the percentage of complement fixing antibodies in red kangaroos was as high as 54%. Pope et al. (Pope, Scott et al. 1960) successfully isolated *C. burnetii* from the blood of one grey kangaroo and 13 of nearly 3,000 kangaroo ticks (*Amblyomma triguttatum*) collected in the study. Interestingly, four of the 13 ticks from which *C. burnetii* was isolated were found on goats and sheep. *Amblyomma triguttatum* requires three different hosts to develop to maturity and thus Pope et al (1960) proposed that the tick may be responsible for the transfer of *C. burnetii* between host species. The authors also concluded from these data that a natural kangaroo-tick cycle existed.

In other animals it has been shown that for several days to months following parturition *C. burnetii* is shed in milk, urine and faeces and thus it is during this period that naïve animals are most at risk of being infected (Berri, Souriau et al. 2001; Arricau Bouvery, Souriau et al. 2003). Parturition in Western grey kangaroos occurs approximately in February (Dawson 2002) but spread of infection from kangaroo birth products is unlikely because products of parturition are minimal in quantity. However, animals may have suppressed immune systems around this time which may increase their susceptibility to infection via the faecal-oral route. This mode of infection may be facilitated by increased rainfall in April/May, which leads to a proliferation of new green feed. This is because sudden change in the composition of feed being ingested by kangaroos may result in decreased faecal consistency and disruption of the natural gut flora of the kangaroos which may reduce their intrinsic resistance to enteric pathogens. This theory is supported by preliminary data on the prevalence of *Salmonella* in the faeces of the same kangaroos included in this study (Bestall 2008) and by a time-series analysis of Q fever in French Guiana which found a strong correlation between rainfall and disease incidence with a lag period of one to three months (Gardon, Heraud et al. 2001). Alternatively, the explosion in arthropod populations that occurs after heavy rainfall (Mushi, Binta et al. 2003) may facilitate vector-borne transfer of coxiellae.

On the three occasions that dams and their pouch young were sampled and tested serologically there was no evidence that the young had been exposed to *C. burnetii*. This might indicate that kangaroos do not shed coxiellae in milk but given the small sample size it is not possible to draw any conclusions with confidence.



#### **6.4.5 Testing kangaroo faecal samples for the presence of *Coxiella burnetii* DNA using a quantitative polymerase chain reaction**

It was surprising that the level of agreement between the results from testing samples from kangaroos with the ELISA and JB153-3 qPCR was much lower than the results obtained from ruminant samples. Fundamental differences in host biology may help to explain the differences observed. However, it is not possible to confirm them without undertaking a histopathological study of tissues from kangaroos that are confirmed to be infected with *C. burnetii*. Sanchez et al. (2006) found that *C. burnetii* was detectable by PCR in mammary gland and lung, but not in liver or spleen eight days after abortion in a group of experimentally infected goats. These animals continued to shed *C. burnetii* in vaginal secretions and milk 26 and 30 days post abortion respectively (Sanchez, Souriau et al. 2006). These results may indicate that during pregnancy coxiellae proliferate and invade several tissue types but following parturition the pathogen may retreat to tissues that are conducive to its dissemination. Conducting similar experiments in kangaroos, in combination with histopathology, would allow the natural transmission cycle to be defined, which will help determine the role of marsupials as reservoirs of *C. burnetii*.

#### **6.4.6 Conclusions**

In other livestock species there is considerable variation in data linking season with outbreaks of disease (Enright, Franti et al. 1971; Yanase, Muramatsu et al. 1997; Raoult, Tissot-Dupont et al. 2000; Hellenbrand, Breuer et al. 2001). There has been no defined link between season and outbreaks of Q fever in humans in Australia (McKelvie 1980; Garner, Longbottom et al. 1997). The lack of agreement between studies related to the seasonal variation of Q fever between and within

countries indicates that not all contributing factors have been identified. In Cyprus, a geographic information system-based approach was used to examine the relationship between human and ruminant seropositivity to *C. burnetii*. However, the analysis revealed that the prevalence in the human population was greater than that found in local ruminants suggesting that there could be another significant reservoir of Q fever in Cyprus (Psaroulaki, Hadjichristodoulou et al. 2006). The importance of a wild reservoir to the dissemination of *C. burnetii* was also highlighted in a study of Q fever in French Guiana where wind and livestock movements were found to be insufficient to account for the exposure distribution observed (Gardon, Heraud et al. 2001). Because different ELISA assays, incorporating different antigens, were used for ruminants and kangaroos it is not possible to make direct comparisons between the two data sets. However, current serological findings of the seroprevalence of *C. burnetii* in Australian ruminants cannot account for all cases of transmission of Q fever. Further work is required to elucidate the role that a putative wildlife reservoir plays in transmission to both domestic animals and humans.

## 7. General Discussion

*Coxiella burnetii* is a significant pathogen of humans but, perhaps due to its ubiquity in all geographic locations, there has been no effort to develop integrated detection and control measures in the livestock production industry. The main goal of this study was to develop a highly sensitive, robust and specific quantitative polymerase chain reaction assay which was compatible with environmental samples associated with domestic ruminants and native Australian marsupials. The resulting qPCR assay system, including a DNA purification method, targeting the repetitive *IS1111a* element was shown to be extremely sensitive, specific and reproducible under laboratory conditions for the detection of *C. burnetii*.

This is the first time an ELISA has been successfully used to detect anti-*C. burnetii* antibodies in kangaroos. The ELISA also appears to be compatible with bandicoot serum and thus will be an important tool for future surveillance of *Coxiella* infections in Australian marsupials. The results of this research also suggest that domestic ruminants may not be the most significant reservoir of *C. burnetii* in Western Australia and that kangaroos may pose a significant threat for zoonotic transfer of this pathogen, particularly for people associated with the kangaroo meat industry. Further research is required to adequately define the significance of this threat in WA and the Eastern states of Australia and to help understand the epidemiology of *Coxiella* infections in marsupials.

A cell culture/qPCR-based assay system to determine the efficacy of disinfectants against *C. burnetii* was optimised and validated. This assay system provided quantitative results with a wide dynamic range and was compatible with both physical and chemical agents. The results showed that sodium hypochlorite had high efficacy against *C. burnetii*, a finding which is at odds with published

data. These findings need to be confirmed using phase I cells and animal inoculation before they can be accepted as valid but it appears likely that treatment of liquid waste with sodium hypochlorite is a viable method of reducing the load of *C. burnetii* in such matrices.

An RNA extraction and quantitative reverse transcription PCR system for detection of gene expression by *C. burnetii* in soil samples was developed to provide a method for the assessment of viability. This method was able to detect transcriptional activity in *C. burnetii* cells added to soil for at least 35 days. However, the correlation between detection of the presence of RNA and the actual viability of a cell is not always perfect and thus results yielded by this type of test should be interpreted with caution. This is particularly true with an organism such as *C. burnetii* that can be metabolically dormant and remain viable and infectious in the environment for long periods of time. Furthermore, the results from this study need to be validated against the gold standard viability assay for *C. burnetii*, animal inoculation, and be reassessed with the virulent phase I form of the organism.

Results from testing faecal or urine samples from livestock and kangaroos showed that interpretation of the PCR results may be confounded by contamination with extraneous DNA. The source of the contamination was most likely the phase II *C. burnetii* that was cultured for other experiments. A qPCR targeting a redundant gene, *JB153-3*, which is deleted in phase II strains, was developed to clarify the classification of test results for all samples. However, the *JB153-3* qPCR is potentially less sensitive compared to the *IS1111a* qPCR because it is less abundant in the *C. burnetii* genome. This may explain why the proportion of faecal samples from ruminants and kangaroos was approximately 75% lower when the *JB153-3* PCR compared to the *IS1111a* qPCR.

The lack of agreement observed between qPCR and serological results appeared to be a function of the cryptic nature of shedding that occurs during *C. burnetii* infection and serves to highlight that neither test can provide categorical infection status data when used in isolation. A strong association was observed between seropositivity of kangaroos and the months of June and July, a trend which may be related to the first significant rainfall of the year. However, a corresponding increase in qPCR-positive samples in May was not observed as would be expected given that it may take as long as 30 days for a detectable IgG response to develop after initial infection.

Overall, this project has developed tools to improve the surveillance for *C. burnetii* in livestock-associated industries while also providing a means to assess the efficacy of disinfectants in the liquid waste these industries produce. Used as an integrated suite of technologies, these tools can provide the livestock industry with the means to assess the risk of infection posed by *C. burnetii* and to implement control strategies which have quantifiable efficacies. Informed risk-management can thus be undertaken that can aid in-house occupational health and safety programs and be used as evidence for Government regulators. During the development of these tools it was revealed that a commonly used industrial disinfectant may be efficacious against *C. burnetii* and also that native Australian marsupials may be a significant wild reservoir of coxiellae. Both of these findings have implications for individuals who come into contact with domestic ruminants and native marsupials and will hopefully lead to new research into the epidemiology of Q fever in Australia.

## 8. Appendices

### Appendix A OD values and percentage reduction in OD value following addition of blocking agents for kangaroo serum samples tested with an ELISA

Sample ID	Absorbance		
	No secondary Ab blocking	With secondary Ab blocking	% reduction in Abs
1265	1.6	1.2	20.8
1266	0.6	0.4	32.9
1268	0.7	0.6	21.2
1269	1.3	1.1	20.1
1270	1.3	1.1	19.4
1271	1.4	1.2	16.3
1272	1.5	1.1	23.0
1273	1.4	1.2	18.6
1274	0.8	0.6	32.7

1275	1.5	1.3	16.2
1276	1.5	1.1	25.4
1277	1.0	0.7	27.5
1278	2.0	1.5	25.7
1279	1.8	1.3	29.8
1280	0.8	0.5	39.2
1281	1.6	1.0	37.9
1282	0.8	0.4	47.5
1283	0.9	0.5	41.6
1284	1.3	0.9	33.4
1285	1.2	0.9	25.3
1287	0.7	0.4	34.6
1407	0.8	0.6	24.4
1408	0.9	0.5	50.9
1409	0.6	0.3	45.8

1410	0.9	0.5	41.7
1411	0.9	0.5	41.9
1412	1.9	1.5	18.6
1413	0.8	0.6	34.7
1414	1.4	1.0	25.8
1415	0.8	0.6	31.2
1416	0.9	0.5	45.8
1417	1.9	1.5	20.9
1418	0.8	0.5	42.5
1419	1.0	0.5	44.1
1420	1.0	0.5	44.6
1421	0.7	0.5	33.5
1422	0.8	0.5	38.6
1423	1.0	0.8	23.6
1424	1.0	0.5	44.5



1425

1.0

0.8

15.8

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**Appendix B Summary of *com1* gene sequencing results from both ruminant and kangaroo samples**

Sample	Species	BP	Match	Accession	Max. ID	% Cov.*
90	bovine	442	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
159	bovine	444	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
175	bovine	447	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	99
189	bovine	456	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
190	bovine	449	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	99
193	bovine	376	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	99
200	bovine	448	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
203	bovine	332	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	99
249	bovine	440	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
348	ovine	361	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	99
366	ovine	369	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	100
367	ovine	450	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100

379	ovine	460	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1202	kangaroo	389	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1263	kangaroo	-	unsuccessful			
1275	kangaroo	460	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1348	kangaroo	389	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1356	kangaroo	444	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	99
1363	kangaroo	460	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	99
1374	kangaroo	399	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1412	kangaroo	376	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	99
1427	kangaroo	411	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1437	kangaroo	458	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1458	kangaroo	401	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1467	kangaroo	368	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	100
1503	kangaroo	442	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100
1585	kangaroo	377	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	99

1607	kangaroo	396	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	99	99
1608	kangaroo	418	<i>C.b</i> Dugway	<a href="#">CP000733.1</a>	100	100

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**\*"% Cov" refers to Percent Coverage, or the percentage of nucleotides in the reference sequence that are covered by the query sequence.**

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## Q-FEVER SUMMARY

Paired kangaroo faecal and serum samples have been collected on a regular basis from a total of five different geographical areas in Western Australia; Capel, Badgingarra, Eneabba, Preston Beach and Manjimup (and surrounds). Samples were collected between May 2007 and December 2008. In total, 1007 paired samples have been tested during this time. Overall results for the areas combined are reported in *Table 1*.

*Table 1. Overall Results for Areas Combined*

	PCR Pos	PCR Neg	TOTAL
ELISA Pos	25	220	245
ELISA Neg	14	748	762
TOTAL	39	968	1007

Across the five geographical areas, a seroprevalence of 24.3% has been found, based on the statistics reported in *Table 1*. This indicates previous exposure to *C.burnetii* and says nothing about the length of time since infection or whether active shedding is still occurring. *C.burnetii* DNA was detected in 3.9% of faecal samples tested.

*Table 2*. reports individual seroprevalence and DNA detection results for the five geographical areas separately.

*Table 2. Analyses of Separate Geographical Areas*

Location	(n)	Seroprevalence	DNA Positive
Capel	269	11.2%	3.7%
Badgingarra	141	27.7%	4.3%
Eneabba	104	28.8%	2.9%
Preston Beach	55	50.9%	1.8%
Manjimup (& Surrounds)	383	25.1%	5%